Investigation of planctomycetal strategies to thrive

-From cell biology to secondary metabolism-

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„Das Erreichen eines Zieles ist etwas Vorübergehendes, das Auf-dem-Weg-Bleiben eine lebenslange Aufgabe.“

“Achieving a goal is temporary, staying on the track is a lifelong endeavor.”

— Ernst Ferstl

Paranimpfen: Muriel van Teeseling
Timo Kohn

Summary

Planctomycetes are an unusual group of bacteria that comprises several exceptional traits. They belong, together with Verrucomicrobia and Chlamydiae, to the PVC-superphylum. Besides their complex cell biological features, bacteria of the phylum Planctomycetes are of environmental importance and are ubiquitously distributed in both terrestrial and aquatic habitats. However, despite their unusual traits and assumed ecological significance, only little is known about their competitive fitness. One hypothesis for their widespread colonization of host organisms is accounted by their lack of peptidoglycan and the resulting insusceptibility against antibiotics that might be produced by rivaling bacteria. Another hypothesis is the production of antibiotic substances by Planctomycetes to defend their habitats. To test both hypotheses, this thesis focuses on the investigation of the planctomycetal cell biology and secondary metabolism.

While **Chapter 1** provides a general introduction on what is known about Planctomycetes and secondary metabolites, **Chapter 2** revisits the proposed absence of peptidoglycan in Planctomycetes. Using bioinformatic methods, it was shown that planctomycetal genomes do contain all genes required for peptidoglycan synthesis. In addition, peptidoglycan was identified by state-of-the-art light- and electron-microscopic methods. Moreover, peptidoglycan sacculi were isolated, which showed characteristic susceptibility to lysozyme as they disintegrated upon treatment. Ultimately, biochemical analyses attested the key components of peptidoglycan in different planctomycetal species and their isolated sacculi. Taken together, the findings provided strong indications for the presence of peptidoglycan in planctomycetal cell walls. In addition, homologues of beta-lactamase encoding genes were identified in the investigated planctomycetal genomes that could explain the observed beta-lactam antibiotic resistance.

Chapter 3 deals with the potential of Planctomycetes as producers of bioactive compounds. We performed the first comprehensive screen for secondary metabolite related genes in planctomycetal genomes. In addition, the planctomycetal substrate spectrum in respect to their algae/plant related lifestyle was investigated to identify putative triggers that might stimulate the production of secondary metabolites.

Chapter 4 addresses the development of techniques for the utilization of Planctomycetes as producers of bioactive molecules. A set of tools is provided, ranging from cultivation methods for marine and limnic Planctomycetes in chemically defined media within process-controlled bioreactors, to the preparation of crude extracts, their chromatographic screening for antimicrobial activities and bioassay-guided fractionation of the crude extracts.

In **Chapter 5**, a new planctomycetal genus is described and validated by polyphasic taxonomy and physiology approaches. Employing the methods developed in **chapter 4**, we successfully

isolated and structurally elucidated a hitherto unknown quorum-sensing molecule with weak antibiotic activity. Additionally, the quorum-sensing activity was investigated and placed in an ecological context with marine *Roseobacter*.

Finally, in **Chapter 6** the results of the preceding chapters are placed in connection and it is discussed to which extent they may have effect on the planctomycetal success in nature. Furthermore, possibilities for future research as well as conceivable applications for secondary metabolites from Planctomycetes are discussed.

Samenvatting

De Planctomyceten zijn een bijzondere groep bacteriën met meerdere buitengewone eigenschappen. Tezamen met de Verrucomicrobia en de Chlamydiae behoren ze tot het PVC-superphylum. Naast hun complexe celbiologie, zijn bacteriën behorend tot het phylum Planctomyceten aanwezig in zowel terrestrische als aquatische milieus en daar van ecologisch belang. Ondanks de kennis over hun ongewone eigenschappen en de notie van hun ecologisch belang, is de vraag nog onbeantwoord gebleven hoe ze succesvol kunnen zijn in de competitie met andere bacteriële soorten in hun niche. Een eerste hypothese luidt dat ze vanwege de afwezigheid van peptidoglycaan ongevoelig zijn voor antibiotica die door andere bacteriën in dezelfde niche worden geproduceerd. Volgens een andere hypothese zijn het de Planctomyceten zelf die antibiotica produceren om hun niche te verdedigen. Dit proefschrift beoogt deze hypotheses te testen en beschrijft daartoe de studies naar de celbiologie en het secundaire metabolisme van Planctomyceten.

Hoofdstuk 1 introduceert de reeds bestaande kennis over Planctomyceten en secundaire metabolieten. In **hoofdstuk 2** wordt ingegaan op de vermeende afwezigheid van peptidoglycaan in Planctomyceten. Bioinformatische analyse toonde aan dat Planctomyceten wel degelijk alle genen bevatten die nodig zijn voor de synthese van peptidoglycaan. Peptidoglycaan werd vervolgens aangetoond met moderne licht- en elektronenmicroscopie. Daarnaast werden peptidoglycaan sacculi geïsoleerd, die, zoals gebruikelijk voor peptidoglycaan, door lysozym afbreekbaar waren. De sterkste aanwijzing voor de aanwezigheid van peptidoglycaan was dat biochemische analyses belangrijke bouwstenen van peptidoglycaan aantoonden in zowel sacculi als intacte cellen van meerdere soorten Planctomyceten. De detectie van beta-lactamase genen in de bestudeerde genomen, kon verklaren waarom deze Planctomyceten ongevoelig zijn voor beta-lactam antibiotica.

Hoofdstuk 3 behandelt Planctomyceten als mogelijke producenten van secundaire metabolieten. Een diepgaande analyse van de genomen gaf voor het eerst een overzicht van genen betrokken bij secundaire metabolieten in Planctomyceten. Daarnaast werd er onderzocht welke substraten Planctomyceten kunnen gebruiken, rekening houdend met hun symbiotische levensstijl met algen en planten. Hierdoor konden substanties worden beschreven die mogelijkerwijs de productie van secundaire metabolieten stimuleren.

In **hoofdstuk 4** worden technieken ontwikkeld, zodat Planctomyceten gebruikt kunnen worden als producenten van bioactieve moleculen. Zo worden methoden voor de cultivatie van mariene en limnische Planctomyceten in chemisch gedefinieerde media in gecontroleerde bioreactoren beschreven. Daarnaast wordt er uitgelegd hoe celextracten gemaakt worden en hoe hieruit via chromatografie en fractionering potentiële bioactieve moleculen kunnen worden verkregen. Ten slotte wordt uiteengezet hoe deze vervolgens getest kunnen worden op hun activiteit.

In **hoofdstuk 5** wordt een nieuw genus binnen de Planctomyceten beschreven met behulp van 'polyphasic taxonomy' en fysiologische methoden. Gebruikmakend van de in **hoofdstuk 4** ontwikkelde methodiek, kon een tot-nog-toe onbekend quorum-sensing molecuul met zwakke antibiotica activiteit worden geïsoleerd en diens structuur worden opgehelderd. Daarnaast werd de quorum-sensing activiteit verder bestudeerd en met mariene *Roseobacter* in een ecologische context geplaatst.

Ten slotte, voegt **hoofdstuk 6** de resultaten van de voorafgaande hoofdstukken samen en deze resultaten worden bezien in het licht van de effecten die ze hebben op het succes van Planctomyceten in de natuur. Vervolgens worden nieuwe onderzoekslijnen en mogelijke toepassingen voor secundaire metabolieten van Planctomyceten bediscussieert.

General Introduction

We are living in a world dominated by bacteria. They outnumber every other organism numerically and by biomass (Whitman *et al.* 1998). They survive at deep sea hydrothermal vents (He and Zhang 2016) and in eternal ice (Miteva and Brenchley 2005). Most natural environments harbor a stunningly diverse collection of microbial species. Within these communities, they compete for space and resources (Hibbing *et al.* 2010). To live through this ongoing struggle, bacteria established diverse active and passive mechanisms by which they can coexist with or dominate other organisms competing for the same pool of resources. The more we know about these mechanisms the more we can exploit them. One prominent example is the medical utilization of antibiotic substances produced by microorganisms (Alanis 2005). In the 1960s it has often been assumed that the discovery of antibiotics heralded the end of clinical bacterial infections. Following this, the development and exploration of novel antibiotics came to standstill. Only four new classes of antibiotics have been approved as therapeutics in the past four decades (Cooper and Shlaes 2011). Seventy years ago, almost all clinical bacteria were sensitive to antibiotics developed and used at the time (Alanis 2005). Nowadays, the first bacterial strains, mostly belonging to the *Enterobacteriaceae* or *Pseudomonadaceae*, exist that are even resistant to last-resort antibiotics and are thus called 'superbugs' (Breidenstein *et al.* 2011, McKenna 2013). Therefore, finding novel antibiotic lead structures calls for new approaches in terms of screening methods as well as novel microbial sources. Surprisingly, little is known about the functions of antibiotics in natural communities. Their ecological role was assumed for 'warfare' amongst the microbes (Waksman and Woodruff 1940). Hence, potential compounds were evaluated for their effectiveness in inhibiting bacterial growth. Other more recent findings showed that some bacteria produce antibiotics in sub-inhibitory concentrations for other purposes like the induction of biofilm formation (Linares *et al.* 2006). The effects of bioactive molecules present in low environmental concentrations might be dramatically different from those achieved by higher concentrations in laboratory or clinical contexts. In natural settings, the primary function of bioactive molecules, including antibiotics, might be to serve as inter- and intraspecies signals for gene expression in response to environmental cues (Davies 2006, Yim *et al.* 2007, Skindersoe *et al.* 2008, Richards and Melander 2009, Romero *et al.* 2011). Consequently, these findings imply at least two more difficulties in terms of antibiotic discovery. On the one hand, screening for growth inhibition typically requires substance concentrations that might exceed naturally occurring levels by several orders of magnitude (Romero *et al.* 2011). For this reason, bioactive compounds which function at lower concentrations in other ways and might include interesting lead structures, are likely to be overlooked. On the other hand, screening approaches often require artificial laboratory setups, including for example axenic cultivation of microorganisms. Therefore, it is not surprising that the cells do not express many potential secondary metabolites without the naturally diverse and changing array of environmental trigger signals, usually omnipresent in nature by various bacteria and other organisms. As a result, even seemingly well exploited bacterial sources of bioactive molecules

might harbor cryptic and not yet discovered antibiotics (Wenzel and Müller 2009). However, besides focusing on well-known antibiotic producers, also novel organismal sources such as yet unexplored bacterial phyla might be promising targets for the identification of novel lead structures. Classically, these phyla are chosen based on certain criteria of which they share many with known bioactive molecule producing bacterial groups. Under this consideration, one potential novel resource might be the bacterial phylum Planctomycetes.

Planctomycetes

Phylogenetic Classification

The first species of the phylum Planctomycetes, *Planctomyces bekefii*, was described by Gimesi in 1924. Because of its unusual round morphology and long stalk-like appendages, *P. bekefii* cells were at first mistaken for fungi (Gimesi 1924). In the following decades, similar organisms, identified as bacteria, were observed by Henrici and Johnsen in 1935 and Zarvazin in 1961 until the first planctomycete was brought into pure culture by Staley in 1973 (Staley 1973). Following this, the number of cultivated Planctomycetes increased steadily (Schmidt 1978, Franzmann and Skerman 1984, Hirsch and Müller 1985, Fuerst *et al.* 1991, Fuerst *et al.* 1997, Strous *et al.* 1999). Planctomycetes were at first linked through phylogenetic analysis with the Chlamydiae (Weisburg *et al.* 1986). Both phyla were later joined along with the Verrucomicrobia, the Lentisphaerae and the OP3-group to the PVC-superphylum (Wagner and Horn 2006). Since the first classification of Planctomycetes based on 16S rRNA gene analyses, where the bacterial order Planctomycetales and family *Planctomycetaceae* were proposed (Schlesner and Stackebrandt 1986), the phylum has undergone several changes and additions. These days, the phylum Planctomycetes is divided in the classes Planctomycetia and the Phycisphaerae which can be further distinguished into the orders Planctomycetales (Ward 2010a, Ward 2010b, Ward 2010c), Phycisphaerales (Fukunaga *et al.* 2009), Brocadiales (Jetten *et al.* 2010) and most recently, Tepidisphaerales (Kovaleva *et al.* 2015). Also, only recently, novel family structures were introduced (Kovaleva *et al.* 2015, Kulichevskaya *et al.* 2015, Kulichevskaya *et al.* 2016) by which the members of the genus *Planctomyces* were reclassified based on genome sequence comparison and 16S rRNA gene identity. In this context, the genus *Planctomyces* was split into the novel genera *Gimesia*, *Planctopirus* and *Rubinisphaera*. However, the initial genus *Planctomyces* with the yet uncultured *P. bekefii* as type species remained (Scheuner *et al.* 2014).

Occurrence of Planctomycetes in the Environment

Planctomycetes have been overlooked in environmental studies for many years, primarily due to mismatches with oligonucleotide primer, formerly used to amplify 16S rRNA sequences (Vergin *et al.* 1998, Arnds *et al.* 2010,). Since then, research on Planctomycetes has progressed tremendously and investigations of microbial communities by modern sequencing approaches rather suggested an ubiquitous presence of Planctomycetes in the environment. They were detected in a wide range of ecosystems where they can be found as free-living cells in various types of soil (Borneman and Triplett 1997, Derakshani *et al.* 2001, Buckley *et al.* 2006), freshwater (Fuerst 1995, Strous *et al.* 1999, Pollet *et al.* 2011) and marine habitats (Kuypers *et al.* 2003, Kirkpatrick *et al.* 2006, Woebken *et al.* 2007, Pizzetti *et al.* 2011b) including marine snow (DeLong *et al.* 1993, Fuchsman *et al.* 2012) and cyanobacterial blooms (Pizzetti *et al.* 2011a, Cai *et al.* 2013). Furthermore, they were also found to live associated with eukaryotic organisms like sponges (Webster *et al.* 2001, Sipkema and Blanch 2010, Izumi *et al.* 2013), prawns (Fuerst *et al.* 1991, Fuerst *et al.* 1997), or periphyton communities on for instance crab shells (Kohn *et al.* 2016), on micro and macro algae (Fukunaga *et al.* 2009, Bengtsson and Øvreås 2010, Bondoso *et al.* 2014, Lage and Bondoso 2014, Yoon *et al.* 2014, Bondoso *et al.* 2015), in termite guts (Köhler *et al.* 2008), with protists (Lage 2013) and also in the foregut of dromedary camels (Samsudin *et al.* 2011). This wide range of associations might be facilitated by, the for multiple planctomycetal species described, stalk- and holdfast structures (Ward 2010b). These holdfast structures enable Planctomycetes to form biofilms on the above-mentioned highly competitive habitats where they have been found to be at times the dominant species. Dominant Planctomycetes populations were found associated with the marine alga *Laminaria hyperborean*, where they made up 50% of the attached-living bacterial community (Bengtsson and Øvreås 2010). Biofilms on marine algae have been described to have mutualistic as well as opportunistic character, where microorganisms protect the alga from fouling-inducing organisms or enable a substrate exchange of for example vitamins and fixed-carbon, but also exploit the rich repertoire of substrates on the algal surface (Hao *et al.* 2010, Ramanan *et al.* 2016). In the environment Planctomycetes seem to live in cooperation with their hosts. However, recently Planctomycetes came into focus of medical issues as opportunistic pathogens after planctomycetal DNA was found in blood samples of leukemia patients (Drancourt *et al.* 2014). But on the other hand, they were also found to be part of healthy human ecto- flora where the presence of these bacteria may help maintain good ecology for other microorganisms (Hannigan and Grice 2013, Somboonna *et al.* 2017).

Physiology

Apart from the exceptional anammox Planctomycetes, members of the Brocadiales, which are chemolithoautotrophic and generate energy by the anaerobic oxidation of ammonium (Strous *et al.* 1999), most members of the phylum Planctomycetes are typically chemoheterotrophic microorganisms, generating energy by the utilization of organic compounds from their environment. Genome analyses of marine Planctomycetes in particular revealed a multitude of sulfatase encoding genes (Glöckner *et al.* 2003, Woebken *et al.* 2007, Wegner *et al.* 2012), which enable Planctomycetes to utilize sulfated heteropolysaccharides that are present in large quantities in marine environments as they are produced by their algal hosts or can be trapped in marine snow (Woebken *et al.* 2007, Bondoso *et al.* 2017). Planctomycetes have been shown to utilize a variety of carbon-rich biopolymers (Schlesner *et al.* 2004, Izumi *et al.* 2013) like cellulose (Schellenberger *et al.* 2010, Pepe-Ranney *et al.* 2016) and pectin (Ivanova *et al.* 2016). Species of the Phycisphaerales are even able to use gelating agents of algal origin, such as agar, a medium component usually used in bacterial cultivation (Fukunaga *et al.* 2009; Yoon *et al.* 2014; Kovaleva *et al.* 2015), for growth. Planctomycetes-specific media in general contain only low amount of carbon and nitrogen sources (Giovannoni *et al.* 1987a, Lage and Bondoso 2012). A common ingredient in Planctomycetes-selective media is *N*-acetylglucosamine that has been shown to be sufficient as sole carbon and nitrogen source to support planctomycetal growth (Schlesner 1994, Lage and Bondoso 2012). This and the natural occurrence of Planctomycetes in niches where these compounds are predominant, has led to the hypothesis that Planctomycetes play an important role in the carbon conversion cycle (Woebken *et al.* 2007, Izumi *et al.* 2013).

Cell Morphology

Since records on Planctomycetes began, they were characterized as bacteria with unusual cell biological features. They were described as spherical, rosette-forming cells, connected by non-cellular stalks and using a budding mechanism for cell division (Figure 1 A), previously only known for yeasts (Gimesi 1924, Fuerst 1995). Nowadays spherical to pear-shaped forms are known for this phylum (Staley 1973, Franzmann and Skerman 1984,). Some species also divide by binary fission (Fukunaga *et al.* 2009, van Niftrik *et al.* 2009, Yoon *et al.* 2014, Kovaleva *et al.* 2015). Before genetic analyses became accessible, morphological characteristics were used to identify Planctomycetes. One hallmark feature are their crateriform structures (Figure 1 A), found on planctomycetal cell surfaces with an often even distribution pattern (Arnds *et al.* 2010). Stalk formation, mostly at the cell poles, but also fibre-like appendages resembling bacterial pili emerging from these crateriform structures (Figure 1) were observed (Starr *et al.* 1983, Jogler *et al.* 2011). The function of those structures stayed elusive until fairly recently uptake of macromolecules has been demonstrated and a role in nutrient uptake for the crateriform structures has been suggested (Boedeker *et al.* 2017).

Cell Division

All known planctomycetal species, whether budding or dividing by binary fission, share the unique characteristic that cell division takes place without the common cell division protein FtsZ (Angert 2005). Since no FtsZ exists in Planctomycetes, other mechanisms and proteins have to be involved. Candidate proteins with potential roles in planctomycetal budding division were identified employing comparative analysis on full genome sequences (Pilhofer *et al.* 2008, Jogler *et al.* 2012). For the anammox planctomycete *Candidatus* Kuenenia stuttgartiensis, which divides by binary fission, it was shown that the protein KustD1438 most possibly replaces FtsZ (van Niftrik *et al.* 2009). However, comparison of full genome sequences of non-anammox Planctomycetes revealed no *kustd1438* homologue, suggesting that this gene is exclusively present in the order Brocadiales (van Niftrik *et al.* 2009, Jogler *et al.* 2012).

Planctomycetal Cell Plan

The planctomycetal cell plan is under heavy debate. Historically, the cytoplasm was suggested to be separated by an intracytoplasmatic membrane into two compartments, the inner pirellulosome and the outer paryphoplasm. In this scenario, the for bacteria untypical nucleus-like condensed DNA (nucleoid) (Figure 1 B) alongside with ribosomes and cellular RNAs is localized within the inner pirellulosome. These compartments are thought to be surrounded by an outermost membrane, suggested as the cytoplasmic membrane (Lindsay *et al.* 1997, Lindsay *et al.* 2001, Fuerst and Sagulenko 2011, Sagulenko *et al.* 2014). However, more recently the outermost membrane was proposed to be a rather normal Gram-negative outer membrane (Speth *et al.* 2012). Different independent studies came to similar results and consequently, the paryphoplasm should be interpreted as a periplasm, the intracytoplasmic membrane as a cytoplasmic membrane and the pirellulosome as the cytoplasm (Figure 1 B) (Santarella-Mellwig *et al.* 2013, Devos 2014a, Devos 2014b, van Teeseling *et al.* 2015, Boedeker *et al.* 2017). Nevertheless, the cytoplasmic membrane is highly curved, leaving a variable, often large, periplasmatic space in between cytoplasm and outer membrane (Figure 1 B) (Boedeker *et al.* 2017). The presence of the additional membrane-bound compartment, the anammoxosome, in anammox Planctomycetes stays beyond debate (Lindsay *et al.* 2001, Niftrik *et al.* 2004, Jetten *et al.* 2010). For a long time, another remarkable feature of Planctomycetes was the early proposed lack of a peptidoglycan (PG) in planctomycetal cell walls (König *et al.* 1984, Liesack *et al.* 1986). All known free-living bacteria possess PG for maintaining their cell shape and to withstand osmotic challenges arising from environmental influences. PG is a rigid polymeric mesh of linear glycans (Lovering *et al.* 2012) cross-linked via short peptides (Vollmer and Holtje 2004). During an in-depth analysis the PG key components, the non-proteinogenic 2,6-diaminopimelic acid, which serves as trifunctional linker of the PG glycan strands (Desmarais *et al.* 2013) and *N*-acetylmuramic acid, one of the two sugar components forming the glycan backbone, were not detected in Planctomycetes. Instead it was proposed that Planctomycetes contain a cell

wall rich in glycoproteins (König *et al.* 1984, Liesack *et al.* 1986). This finding was further endorsed, as a distinct PG-layer seemed absent from ultrathin sections studied with transmission electron microscopy (Giovannoni *et al.* 1987b, Fuerst and Webb 1991, Lindsay *et al.* 2001, Bondoso *et al.* 2011). In addition, the genome sequences of several Planctomycetes apparently lacked a varying amount of genes necessary for the biosynthesis of PG (Glöckner *et al.* 2003, Strous *et al.* 2006, Jogler *et al.* 2012, Guo *et al.* 2014). Another main argument for the absence of PG has been the finding that all tested Planctomycetes were resistant to beta- lactam antibiotics targeting PG biosynthesis (Fuerst and Webb 1991, Schlesner *et al.* 2004, Fukunaga *et al.* 2009, Kulichevskaya *et al.* 2009, Cayrou *et al.* 2010,). An alternative cause for the observed resistance, the production of beta-lactamases, was ruled out for Planctomycetes (Claus *et al.* 2000).

Cell Cycle

A key feature of Planctomycetes bacteria is the complex life style. In laboratory liquid cultures or natural aquatic environments, Planctomycetes perform a so-called life-cycle-switch, which describes the morphological change between a sessile life style as mostly stalked cells and a motile life style as planktonic swimmer cells (Figure 1 A) (Tekniepe *et al.* 1981, Hirsch and Müller 1985). As it was shown for *Planctopirus limnophila* (formerly known as *Planctomyces limnophilus*), cell division is restricted to the stalked cells (Hirsch and Müller 1985). The reproduction phase starts by first developing a holdfast structure and stalk, attaching to a surface, and then budding off flagellated cells (Tekniepe *et al.* 1981, Tekniepe*et al.* 1982). Sessile cells in biofilms represent only one phase in the planctomycetal life cycle. The mechanism how planktonic swimmer cells 'decide' to enter the developmental program of holdfast and stalk formation remains elusive. However, employing comparative genomics it was shown, that their compartmentalization and life cycle are associated with a complex signal transduction repertoire (Jogler *et al.* 2012).

On the one hand, this complexity might be the reason why Planctomycetes belong to the so-called slow-growing organisms (Strous *et al.* 1998), which makes them susceptible to be outgrown by faster growing bacteria or fungi. But as mentioned above, Planctomycetes even dominate highly competitive carbon rich habitats. So on the other hand, this complexity might be key to their success as their different signaling logic along with presumable novel secondary metabolites might include yet unknown bioactive molecules that await discovery.

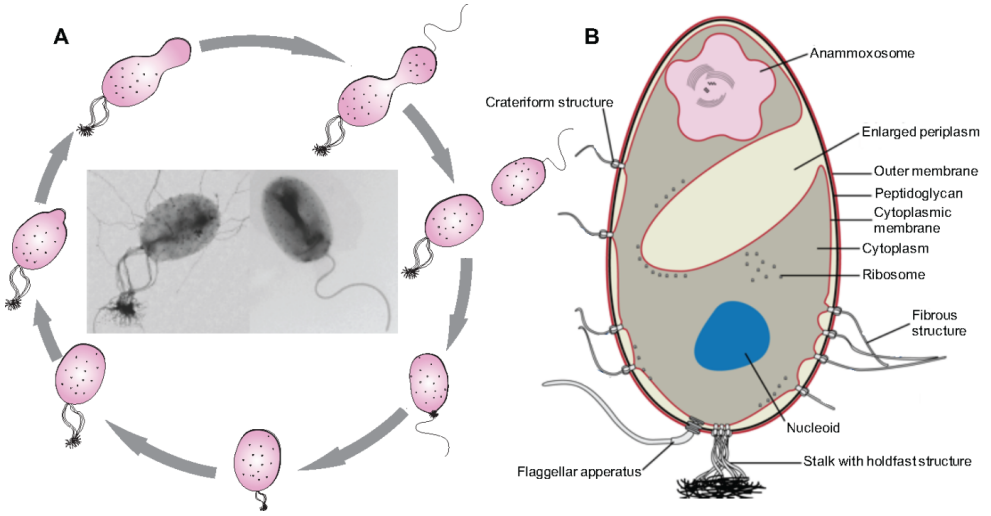

Figure 1: Cartoon of Schematic Appearance of Planctomycetes.
A: displays a schematic budding cell division cycle and an electron microscopy of a pear-shaped Planctomycete. Crateriform structures are evenly distributed on the cell surface. **B:** displays schematically the summarized cell plan of Planctomycetes possessing a Gram-negative cell architecture with an invagination of the cytoplasmic membrane. Crateriform structures are found at sites of contact between the inner and outer membrane (modified with permission from (Boedeker *et al.* 2017)).

The Bacterial Metabolism

The bacterial metabolism is divided into anabolism and catabolism (Doelle 1975). While anabolism represents biosynthetic reactions that lead to the buildup of cell material, the end products of catabolism are reassembled to form primary intermediates such as amino acids, nucleotides, vitamins, carbohydrates, and fatty acids. Metabolites, the intermediates and products of metabolism, are typically characterized by small molecules with various functions. They can be categorized into primary and secondary metabolites. The products of primary metabolism serve basic, often essential roles in support of growth and reproduction (Vining 1992). Primary metabolites are typically formed during the growth phase. On the other hand, secondary metabolites are predominantly formed during the end of the growth phase or near the onset of the stationary phase. They are typically organic compounds produced through the modification of primary metabolite synthases. Such compounds appear to be of secondary importance to the organism and are apparently not essential for growth (Vining 1992).

Bacterial Secondary Metabolites

The common feature of the many known secondary metabolites is their survival value for the producing organism (Vining 1992). Secondary metabolites were reported to mediate symbiosis between bacteria (Piel 2009) but also interfere various eukaryotic organisms *e. g.* the above-mentioned interaction with algae (Ramanan *et al.* 2016). Moreover, they are acknowledged as signaling molecules either for quorum-sensing or quorum-quenching (Demain and Fang 2000, Dong *et al.* 2001, Dong and Zhang 2005). Thus, they may be seen as facilitators for inter- and intra-species chemical crosstalk. However, the most prominent representatives of secondary metabolites are antibiotics. They are known to serve as competitive weapons used against other pro- and eukaryotes (Demain and Fang 2000). The predisposition to produce these compounds is distributed in all microbial taxa (Vining 1992, Berdy 2005).

Antibiotic Agents

Antibiotic agents are mostly small molecules produced by both pro- and eukaryotic organisms. They revolutionized treatment of infectious diseases. The ability to produce such active molecules is unevenly distributed among different species and seems more closely associated with existence in a competitive environment than with phylogeny (Vining 1992, Berdy 2005). There are hundreds of conserved essential bacterial proteins that could serve as antibiotic target, though the number of currently exploited targets is limited to a small set (Lewis 2013). The most successful antibiotics hit only three bacterial metabolic pathways: the protein biosynthesis facilitated by ribosomes (Wilson 2014), cell wall synthesis (Schneider and Sahl 2010) and DNA synthesis facilitated by DNA gyrase (Maxwell 1997) or DNA topoisomerase (Khodursky *et al.* 1995).

Signaling Molecules

Numerous bacteria are able to monitor their population density and regulate their gene expression accordingly through quorum-sensing (QS), thus the QS-regulated processes deal with multicellular behaviors like microbe-microbe interaction *e.g.* growth and development of biofilm and host-microbe interactions (symbiosis and pathogenesis) (Grandclement *et al.* 2016). The process relies on the production, release, and group-wide detection of specific signal molecules called autoinducers (Nealson 1977, Fuqua *et al.* 1994). However, studies on QS revealed a large diversity of QS signals and their regulated functions (Fuqua *et al.* 1994, Whitehead *et al.* 2001, Jimenez *et al.* 2012, Monnet *et al.* 2016). In addition to their signaling roles, it was shown that QS compounds may act as biosurfactants or feature antibiotic activities and may induce some responses in organisms which do not produce them (Schripsema *et al.* 1996, Kaufmann *et al.* 2005, Lowery *et al.* 2009, Salvucci 2016). On the other hand, disabling QS circuits (quorum-quenching) with small molecules has also been proposed as a potential strategy to prevent bacterial pathogenicity (Dong *et al.* 2001).

Where to Look for 'Talented' Producers

In the kingdom of bacteria, *Actinobacteria* are the best studied antibiotic producing organisms, followed by myxobacteria, *Cyanobacteria* and certain *Bacillus*- and *Pseudomonas* strains (Berdy 2005). However, these bacteria have been exhaustively screened in the past. Unfortunately, as a consequence thereof, the discovery of novel lead structures decreased, while rediscovery rates of known compounds increased (Cooper and Shlaes 2011). Marine microorganisms in general attract continuously more attention in terms as source of new natural products, with 491 new compounds reported in 2013 alone. Whereby marine bacteria originated compounds account for 169 new compounds by itself (Blunt *et al.* 2013). In this connection, especially marine Gram-positive bacteria as the already known talented producer Actinomycetes have obtained the most attention. However, yielding notable compounds such as the acetyl-CoA carboxylase-inhibiting antibiotics moiramides and the andrimids (Isnansetyo and Kamei 2009), as well as the QS molecule AI-2 (Mansson *et al.* 2011) or the tyrosinase inhibitors thalassotalic acids (Deering *et al.* 2016) from marine γ-proteobacteria have raised awareness to the biosynthetic potential of marine Gram-negative bacteria in general. Moreover, recognition that marine Gram-negative bacteria produce such molecules as the antiviral and antitumor depsipeptides didemnins (Xu *et al.* 2012), and quite likely the macrolide lactones bryostatins (Sudek *et al.* 2007), support the assumption for discovery of molecules with biomedical value. This awareness is furthermore encouraged through genome mining based and isolation-driven discovery approaches (Still *et al.* 2014, Machado *et al.* 2015). These and other studies portend the future success of discovery efforts aimed at marine Gram-negative bacteria. The chance to discover yet unknown structures correlates among other factors directly with the phylogenetic distance between the microorganism under study and the already known producers (Müller and Wink 2014). Thus, phylogenetically distinct bacterial lineages might represent a valuable source for novel secondary metabolites. Besides such phylogenetic aspects, most potent antibiotic producers are characterized by large genomes with often more than 8 Mb and complex life styles, involving for example differentiation processes (Müller and Wink 2014, Tracanna *et al.* 2017). Employing these criteria, entire bacteria phyla could be judged as putative 'talented' producers if basic knowledge about their ecology, cell biology and genomic architecture is available.

Planctomycetes Potential as 'Talented' Producers

Planctomycetes meet certain of the above-described criteria. Firstly, they are ubiquitous and environmentally important bacteria, possessing large genomes (Glöckner *et al.* 2003). Secondly, the planctomycetal cell biology is remarkable and was speculated to parallel eukaryotic cells in some aspects (Fuerst and Sagulenko 2011). Moreover, traits like their lifestyle switch after cell division, reminds of the cell cycle of Myxobacteria and also their complex transduction repertoire resembles that known for pseudomonads. Planctomycetes were found to possess secondary metabolite gene clusters employing genome mining,

targeting nonribosomal peptide synthetases (NRPS) and polyketide synthases (PKS) encoding genes (Donadio *et al.* 2007). In the same year, planctomycetal type I polyketide keto-synthase domains were identified in two costal Antarctic sediments (Zhao *et al.* 2008). Thus, also first bioinformatic studies support the assumption that Planctomycetes might harbor yet unknown secondary metabolites with potential biomedical value.

Aim and Outline of the Study

The aim of this study is to elucidate how Planctomycetes contrive to be as successful in this variety of natural habitats. For this purpose, two hypotheses are evaluated in more detail.

I. The lack of peptidoglycan makes Planctomycetes insusceptible to antibiotics which are produced by their potential competitors, thus giving them the ability to not only colonize favorable habitats, but also to dominate them.

II. The production of bioactive secondary metabolites enables them to actively protect their habitats and resources against other, faster growing heterotrophic bacteria.

Since the lack of peptidoglycan (PG) is assumed to be a passive mechanism providing Planctomycetes resistance against antibiotics, a closer look on the proposed PG absence is given in **chapter 2**. PG is a stable mesh of linear glycan strands cross-linked via peptides (Vollmer and Holtje 2004, Lovering *et al.* 2012). Despite its simplicity, it enables bacteria to withstand osmotic pressure and to maintain cell shape and integrity of the cytoplasmic membrane. Thus, it can be seen as bacterial hallmark trait. Apart from host-associated Mollicutes, that live in an isotonic environment and presumably lost their PG along with host specialization (Miles 1992, Razin 2006), only members of the PVC-superphylum were proposed to lack PG (König *et al.* 1984, Liesack *et al.* 1986, Pilhofer *et al.* 2008, Fuerst and Sagulenko 2011). Employing state of the art methods, Chlamydiae were recently found to possess PG and a long lasting dispute about the 'Chlamydiae anomaly' was settled (Pilhofer *et al.* 2013). In contrast, the free-living Planctomycetes, that are believed to differ from all other bacteria in terms of their cellular organization, are still assumed to possess a proteinaceous cell wall, instead of PG. However, employing modern bioinformatic, biochemical and microscopic approaches, the presence of PG was proven in the planctomycetal cell wall (Jeske *et al.* 2015). This finding points towards the presence of PG in all free-living bacterial species. However consequently, the question how Planctomycets achieve resistance against beta-lactam antibiotics would stay unanswered, since an alternative cause, the production of beta-lactamase enzymes was ruled out for Planctomycetes (Claus *et al.* 2000). Thus, also the lack of beta-lactamase enzymes in Planctomycetes was revisited bioinformatically and found to be mistaken. All investigated planctomycetal genomes could be identified to harbor at least one beta-lactamase homologue (Jeske *et al.* 2015).

In both cases, the identification of peptidoglycan and beta-lactamases, computational approaches played crucial roles in clarification, indicating that information, obtained by genome analyses, might be key to inquire the second hypothesis. Accordingly, **chapter 3** sets the main focus on bioinformatic investigation of planctomycetal genomes and to answer the question if they harbor secondary metabolite genes and thus have the potential to produce bioactive compounds. Herein the first comprehensive screen for secondary metabolite related genes in planctomycetal genomes was performed. As a result, within the 15 analyzed genomes 112 genes or gene clusters were identified. However, it is well-known that bioactive small molecules are frequently produced only due to environmental cues (Straight and Kolter 2009, Seyedsayamdost *et al.* 2011). Hence, those identified genes might be silent under axenic laboratory conditions (van Wezel and McDowall 2011, Gomez-Escribano and Bibb 2012, Yamada *et al.* 2012). Since Planctomycetes have been shown to be associated with eukaryotes, such as water plants or algae (Lage and Bondoso 2011, Matsuzawa *et al.* 2010), we accordingly optimized a high-throughput assay based on the Phenotype MicroArray technology. Thus, the planctomycetal substrate spectrum in respect to their algae/plant related lifestyle was investigated, to identify putative triggers that might stimulate secondary metabolite production.

As the potential to produce secondary metabolites has been demonstrated for Planctomycetes, further investigation calls for techniques to utilize Planctomycetes. Thus, **chapter 4** is primarily concerned with the development of such methods for Planctomycetes. A set of tools is established ranging from cultivating marine and limnic Planctomycetes in chemical defined media within process-controlled bioreactors, to the preparation of planctomycetal extracts, their chromatographic screening for antimicrobial activities and bioassay-guided fractionation of the crude extracts. Moreover, we were able to assign the first antibiotic activity related to planctomycetal antimicrobial compounds. In conclusion, we developed a large-scale cultivation procedure to allow future structural elucidation of such compounds.

Employing this methodology in **chapter 5** enabled the isolation and structural elucidation of the first secondary metabolites derived from Planctomycetes. The compounds were isolated from Mal15^T, a new planctomycetal species which is described by a polyphasic taxonomy and physiologic approach in this chapter. The produced main compound (stieleriacine A_1) is suggested as a yet unknown QS molecule. At low concentrations, determined as physiological concentrations, stieleriacine A_1 seems to accelerate the interspecies shift from motile cells to reproducing cells. Whereas at high concentration (100-fold), stieleriacine A_1 exhibits antibiotic activity. Physiologic concentration showed no effect in other bacterial cultures. However, the 100-fold concentration led to significant changes in biofilm formation of two different marine Roseobacter strains. Strikingly, the presence of stieleriacine A_1 reduces the biofilm formation ability of *Sulfitobacter dubius* but enhances biofilm formation of *Phaeobacter inhibens*.

In **chapter 6** an integrative overview and discussion of the results, obtained in the preceding

chapters, is given. The results are placed in connection with the above stated hypotheses. Moreover, possibilities for future research as well as conceivable applications for secondary metabolites from Planctomycetes are discussed. With the new results of **chapter 2** we were able to disprove the hypothesis of peptidoglycan-less Planctomycetes. However, based on the results of **chapter 3** and **4** we reinforced the hypothesis of Planctomycetes being a promising source for novel secondary metabolites with antimicrobial function. Moreover, results of **chapter 5** indicate that there might be a further quorum-sensing function for the secondary metabolites isolated from Planctomycete Mal15^{T}.

Planctomycetes do Possess a Peptidoglycan Cell Wall

This chapter has been published as:

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ABSTRACT

Most bacteria contain a peptidoglycan (PG) cell wall, which is critical for maintenance of shape and important for cell division. In contrast, Planctomycetes have been proposed to produce a proteinaceous cell wall devoid of PG. The apparent absence of PG has been used as an argument for the putative planctomycetal ancestry of all bacterial lineages. Here we show, employing multiple bioinformatic methods, that planctomycetal genomes encode proteins required for PG synthesis. Furthermore, we biochemically demonstrate the presence of the sugar and the peptide components of PG in Planctomycetes. In addition, light and electron microscopic experiments reveal planctomycetal PG sacculi that are susceptible to lysozyme treatment. Finally, cryo-electron tomography demonstrates that Planctomycetes possess a typical PG cell wall and that their cellular architecture is thus more similar to that of other Gram-negative bacteria. Our findings shed new light on the cellular architecture and cell division of the maverick Planctomycetes.

INTRODUCTION

Dissolved solutes within free-living microorganisms cause significant osmotic pressure that challenges cell integrity. While most archaea rely on S-layers as protective exoskeletons (Engelhardt 2007), bacteria possess peptidoglycan (PG) for maintaining their cell shape. PG is a rigid polymeric mesh of linear glycans (Lovering *et al.* 2012) cross-linked via short peptides (Vollmer and Holtje 2004), whose remodeling -together with FtsZ and other proteins- is essential for septal formation during cell division (Egan and Vollmer 2013). Thus, most bacteria possess both, a PG cell wall and FtsZ, while the PG synthesis proteins and cell division proteins are encoded in the division and cell wall (dcw) operon. While members of the class Mollicutes lack PG, several species encode FtsZ (Razin 2006). However, Mollicutes are osmotically fragile, exhibit plasticity and pleomorphism (Miles 1992), and depend on an eukaryotic host to provide an osmotically stable environment for living. Thus, they are not considered as free-living.

In contrast, cells of the free-living, ubiquitous and environmentally important phylum Planctomycetes were thought to lack PG and FtsZ as well (Pilhofer *et al.* 2008, Fuerst and Sagulenko 2011, Jogler *et al.* 2012), and to possess a proteinaceous cell wall instead (König *et al.* 1984, Liesack *et al.* 1986). Indeed, the dcw clusters show signs of degradation in some Planctomycetes, where some genes are lost while other are present (Jogler *et al.* 2012, Pilhofer *et al.* 2008). The absence of FtsZ may contribute to the unusual polar budding cell division of most Planctomycetes (Fuerst and Sagulenko 2011).

The inability to identify PG formed a cornerstone in the concept of a planctomycetal cell architecture that differs from all other bacteria. It was further proposed that their cytosol is divided by an additional intracytoplasmic membrane system into a paryphoplasm and a pirellulosome (Fuerst and Sagulenko 2011), a concept that has been challenged recently employing bioinformatic and microscopic methods that pointed towards a more Gram-negative-like planctomycetal cell plan (Speth *et al.* 2012, Santarella-Mellwig *et al.* 2013, Devos 2014a,).

The question whether PG is present or absent in Planctomycetes is key for understanding (i) the compartmentalized planctomycetal cell architecture, (ii) the unusual FtsZ-independent cell division and (iii) the planctomycetal endocytosis-like uptake of proteins. Furthermore, this question is of significance in evolutionary terms as Planctomycetes were suggested as a 'missing link' between prokaryotes and eukaryotes (Devos and Reynaud 2010) or as species 'beyond the bacterium (Fuerst and Sagulenko 2011). The postulation of a proteinaceous cell wall dates back three decades (König *et al.* 1984) and has been passed on since then. Despite its conceptual importance, the cell wall composition of Planctomycetes has been rarely addressed experimentally (Liesack *et al.* 1986, Claus *et al.* 2000, Guo *et al.* 2014). However, recent reports on penicillin sensitivity of anammox Planctomycetes questioned their lack of a PG cell wall (Hu *et al.* 2013). Furthermore, employing state-of-the-art methods, Chlamydiae were recently found to produce PG and a long lasting dispute about the

'Chlamydiae anomaly' was settled (Pilhofer *et al.* 2013, Liechti *et al.* 2014,). In this study, we revisit the nature of the planctomycetal cell wall. Employing modern bioinformatic approaches, we demonstrate that planctomycetal genomes harbour the genes required for PG synthesis. In addition, biochemical assays reveal the sugar and peptide components of PG in selected planctomycetal species. Our findings are further supported by light- and electron microscopic experiments that reveal planctomycetal cells and PG sacculi, both being susceptible to lysozyme treatment. Finally, cryo-electron tomography (CET) demonstrates that Planctomycetes possess a cell wall, comparable to that of other Gram-negative bacteria.

MATERIAL & METHODS

Phylogeny

Gene sequences (16S rRNA; Supplementary Table 1) were aligned (SINA Alignment Service (Pruesse *et al.* 2012)) and manually corrected. Maximum Likelihood (RAxML module; rate distribution model GTRGAMMA; rapid bootstrap analysis algorithm), NJ (ARB NJ tool; Felsenstein correction) and Maximum Parsimony (Phylip DNAPARS module) trees were calculated including the *E. coli* 16S rRNA gene positions 63–1,406, employing the ARB software package (Ludwig *et al.* 2004). Bootstrap values were computed with 1,000-fold resampling.

Beta-lactamase Assay

Beta-lactamase production was triggered for 2 h (100 µg/mL ampicillin). Cells were then lysed on ice by sonication (2 x 2 min, 70%; Bandelin, Sonopuls) and pelleted using a centrifuge. Then 50 mL nitrocefin (0.5 mg/mL; Calbiochem) were added to 500 mL of the supernatant. After 30 min incubation in the dark a red colour indicated beta-lactamase activity.

Bioinformatic Analysis of Planctomycetal Genomes

Proteins required for PG synthesis from *E. coli* were used as blastp (Altschul *et al.* 1990) and PSI-BLAST (five iterations (Altschul *et al.* 1997)) query to identify homologues in *Planctopirus limnophila* (formerly known as *P. limnophilus*) and *Rubinisphaera brasiliensis* (formerly known as *P. brasiliensis*). The obtained planctomycetal proteins were then compared against all the other sequenced Planctomycetes (Supplementary Table 1 and 2 and Supplementary Data 2-4). Homologues required a threshold (identity >30% and an *e* value $<1e^{-6}$), conserved domain architecture (Marchler Bauer *et al.* 2011) and a reciprocal blastp match against *E. coli*. Beta-lactamase enzymes (selected based on (Bush 2013), see Supplementary Table 3) were compared against the NCBI database using blastp (Altschul *et al.* 1997) with default settings and 'Planctomycetes' as taxon filter. Homologues were identified using an initial threshold (identity ≥ 20%; coverage ≥ 40%; *e* value ≤ e^{-4}) and a reciprocal blast analysis (identity ≥ 30%; coverage ≥ 45%; *e* value ≤ e^{-6}).

Cultivation Conditions

Unless noted otherwise *Rhodopirellula baltica* SH1 DSM 10527, was cultured at 28 °C in M2 medium composed of 1 g/L peptone, 1 g/L glucose, 250 mL double concentrated artificial seawater (46.94 g/L NaCl, 7.84 g/L Na_2SO_4, 21.28 g/L $MgCl_2 \cdot 6H_2O$, 2.86 g/L $CaCl_2 \cdot 2H_2O$, 0.384 g/L $NaHCO_3$, 1.384 g/L KCl, 0.192 g/L KBr, 0.052 g/L H_3BO_3, 0.08 g/L $SrCl_2 \cdot 6H_2O$, 0.006 g/L NaF), 10 mL vitamin solution (double concentrated) and 20 mL/L mineral salt solution buffered with 5 mM Tris/HCl at pH 7.5. *Planctomyces limnophilus* DSM 3776 and *Gemmata obscuriglobus* DSM 5831 were cultivated at 28°C in M3 medium composed of

1 g/L peptone, 1 g/L yeast extract, 1 g/L glucose, 5 mL vitamin solution (double concentrated) and 20 mL/L mineral salt solution buffered with 10 mM HEPES at pH 7.5. Strain L21-RPul-D3 was obtained from the anoxic zone of a hypersaline microbial mat on the Kiritmati atoll (Central Pacific). Cells of this strain were cultured anaerobically at 35°C for six days in DSMZ medium 1527 in which D-glucose was replaced with 0.5 g/L dextran as carbon source. Mineral salt solution and vitamin solution were prepared according to DSMZ medium 621. The metal salts for preparing mineral salt solution consisted of 250 mg/L Na-EDTA, 1095 mg/L $ZnSO_4 \cdot 7H_2O$, 500 mg/L $FeSO_4 \cdot 7H_2O$, 154 mg/L $MnSO_4 \cdot H_2O$, 39.5 mg/L $CuSO_4 \cdot 7H_2O$, 20.3 mg/L $CoCl_2 \cdot 6H_2O$, 17.7 mg/L $Na_2B_4O_7 \cdot 10H_2O$ of which 50 mL were added per liter of mineral salt solution. *Escherichia coli cells* DSM 498 were grown over night at 37°C in lysogeny broth (LB). *Spirochaeta asiatica* DSM 8901 was cultivated for 2 days in DSMZ medium 1263 at 35°C. For the detection of diaminopimelic acid, P. *limnophilus* was cultivated in 10 mM NH_4Cl, 10 mM KH_2PO_4, 100 mM KNO_3, 200 mM $MgSO_4 \cdot 7H_2O$, 100 mM $CaCl_2 \cdot 2H_2O$, 250 mM $CaCO_3$ and 300 mM $NaHCO_3$, 20 mL/L mineral salts solution and *R. baltica* was cultured in 250 mL/L of 2· artificial sea water (46.94 g/L NaCl, 7.84 g/L Na_2SO_4, 21.28 g/L $MgCl_2 \cdot 6H_2O$, 2.86 g/L $CaCl_2 \cdot 2H_2O$, 0.384 g/L $NaHCO_3$, 1.384 g/L KCl, 0.192 g/L KBr, 0.052 g/L H_3BO_3, 0.08 g/L $SrCl_2 \cdot 6H_2O$ and 0.006 g/L NaF) and 5 mL /L 1M Tris/HCl pH 7.5). Each culture broth was supplemented with 0.25 g/L glucose. *G. obscuriglobus* cells were grown like *P. limnophilus*, but supplemented with 0.25 g/L pullulan instead of glucose. Cultures were incubated at 28 °C for at least 3 days. *S. asiatica* was grown in DSMZ medium 1263 at 35°C for 2 days. For MurNAc and GlcNAc detection *P. limnophilus* medium was supplemented with 0.25 g/L dextran instead of glucose at 28°C for 3 days. *M. vannielii* was cultivated in DSMZ medium 119 at 35°C for 2 days.

Detection of GlcNAc and MurNAc

Cell pellets from 50 mL culture were treated with substrate-specific PG-hydrolysing enzymes (*Streptomyces globisporus* mutanolysin from Sigma, *E. coli* MurNAc-L-alanyl amidase D, AmiD, isolated according to (Uehara and Park 2007), *Bacillus subtilis* *N*- acetylglucosaminidase, *Bs*NagZ, isolated according to (Litzinger *et al.* 2010); 10 mg enzyme were used each, per 40 mL sample) and then incubated with 80 ng of the MurNAc/GlcNAc specific kinase of *Clostridium acetobutylicum*, MurK, isolated according to (Marchler-Bauer *et al.* 2011) and [γ-32P]-ATP (3,000 Bq). Samples were spotted immediately and after 1 h of incubation on a thin-layer chromatography (TLC) plate. The TLC was developed in a solvent mixture of *n*-butanol, methanol, 25% aqueous NH_3 and water (5:4:2:1) and detected after incubation for 12 h using a photosensitive screen (Kodak) on a Typhoon FLA7000 biomolecular phosphoimager (GE Healthcare).

Detection of Diaminopimelic Acid

Cell pellets were obtained from 6–20 mL cultures, lyophilized, hydrolysed (200 µl 4N HCl, 100°C, 16 h) and dried in a vacuum desiccator. Amino-acids derivatized to *N*-heptafluorobutyryl isobutylesters (Schumann 2011) were resolved in ethyl acetate and analysed by GC/MS (Singlequad 320, Varian; electron impact ionization, scan range 60 to 800 *m/z*). The derivatized diaminopimelic acid was detected in Extracted Ion Chromatograms using the characteristic fragment ion set 380, 324, 306 and 278 *m/z* at a retention time of 23.7 min.

Lysozyme Assay

To increase osmotic stress, *P. limnophilus* and *G. obscuriglobus* cells were washed with ddH_2O, while *R. baltica* and L21-RPul-D3 were washed in tap water and artificial sea water, respectively. Cells were incubated with EDTA (20 mM) and lysozyme (10 mg/mL) for up to 48 h at 37 °C and shaking (300 rpm), while lysozyme was omitted in the negative controls. Cells were immobilized (1% agarose-pad) in MatTek 35-mm glass-bottom dishes and imaged under phase-contrast illumination using a Nikon Ti microscope at x100 magnification and the Nikon DS-Ri2 camera employing the Nikon NIS-Elements software.

TEM Analysis of Planctomycetal PG Sacculi.

G. obscuriglobus DSM 5831^T and *P. limnophilus* DSM 3776^T were cultivated in 1 L of respective growth media. Cells were harvested (10 min, 4,000 g, 4°C; JA-14 rotor, Beckman Coulter) and cell pellets were resuspended in 10 mL 10% SDS (w/v) and boiled for 3 h. Sacculi were harvested (30 min, 139,699 g, 4 °C; SW 60 Ti rotor, Beckman Coulter), washed four times in 3mL water and resuspended in 1 mL water supplemented with 0.02% (w/v) sodium azide. Half of the prepared sacculi were incubated with lysozyme (100 mg/mL) for 2 h at 37°C under slight agitation (300 rpm), while lysozyme was omitted in the negative controls. TEM micrographs of murein sacculi were taken after negatively staining with 0.1–2% aqueous uranyl acetate, employing a Zeiss transmission electron microscope EM 910 at an acceleration voltage of 80 kV at calibrated magnifications as previously described (Wittmann *et al.* 2014).

CET of *P. limnophilus* Cells

A late-exponential-phase culture of *P. limnophilus* was gently filtered (10 m membrane filter, Whatman Nuclepore) to remove aggregated cells. Aliquots (3 µL) of the filtered cell suspension were mixed with the same volume of BSA-stabilized 15 nm colloidal gold solution (Aurion) and placed on holey-carbon coated 200 mesh copper grids (R2/1, Quantifoil, Jena, Germany) immediately before thin-film vitrification by plunge-freezing in liquid propane (63%)/ethane (37%). Grids with frozen-hydrated samples were mounted in autogrids (Rigort *et al.* 2012) and ~200 nm thin lamellae of vitrified material were milled with 30 keV gallium ions after the application of a protective platinum layer in a dual-beam (FIB/SEM)

instrument (Quanta 3D FEG, FEI, Hillsboro, OR, USA) equipped with a Quorum cryo-stage maintained at ⁻185°C (PP2000T, Quorum, East Sussex, UK). Milling was carried out at a nominal incident ion beam angle of 22° (15° effectively) using gallium beam currents of 300, 100 and 30 pA in sequential milling steps. Afterwards tomographic tilt series were recorded under low-dose conditions (total dose typically 150 e $Å^{-2}$) on a Titan Krios II (FEI, Hillsboro, OR, USA) equipped with a post-column energy filter and a K2 summit direct electron detector (Gatan, Pleasanton, CA, USA). In the exemplary tomogram (Figure 5) an angular range from ⁻68° to 54° was covered in 2° increments, and the tilt series was recorded at a nominal defocus of ⁻5 µm and a primary magnification of x 33,000 (that is, a pixel size of 0.42nm on the object level). Dose fractionation mode was employed and subframes of each projection were sampled, which were then aligned using custom-made scripts. Estimation and correction of the contrast transfer function was performed for the frame-corrected set of projections according to (Eibauer *et al.* 2012), followed by 3D reconstruction in IMOD v4.7.8, using patch tracking for fine alignment since not enough gold markers were present after sample thinning. Cell wall cross-section averaging for density profile calculation was done on a 3D data set (binned once; 0.85 nm per pixel) as described in (Hoffmann *et al.* 2008). Subframes (90 x 90 pixels) of the cell envelope were extracted from 17 *x*–*y* slices central to the *z*- direction of a tomogram, aligned and averaged. MatLab8 (MathWorks) incorporating the TOM toolbox50 was used for all image processing. Prior to segmentation, the 3D volume was binned three times and filtered by nonlinear anisotropic diffusion in IMOD (*K* value 0.03, 20 iterations). Segmentation in general was done in Amira v5.6.0 with specific automatic membrane segmentation according to (Martinez-Sanchez *et al.* 2011).

RESULTS

Bioinformatic Analysis

We re-analyzed the planctomycetal phylogeny of the 16S rRNA gene sequences from selected species, including the novel, deep-branching, obligate anaerobic and halophilic strain L21-RPul-D3 (Supplementary Table 1), employing Maximum Likelihood, Neighbor Joining and Maximum Parsimony algorithms. The resulting phylogenetic tree (Supplementary Figure 1) guided the selection of suitable model organisms for bioinformatic, biochemical and microscopic analyses, while its long branches might correspond to a high evolutionary divergence of cultivated planctomycetal species (see Supplementary Figure 1 and Supplementary Note 1 for details). In a second step, we employed comparative genomics to analyses planctomycetal genomes with respect to genes required for PG synthesis. Taking the assumed large evolutionary divergence of Planctomycetes into account, we used methods apt to deal with evolutionarily more distant sequences, and analysed entire planctomycetal genomes employing dynamic queries and position-specific iterative BLAST searches (Guo *et al.* 2014) (see Supplementary Note 2 for details). Thus, contrary to a previous study that reported the absence of genes required for PG synthesis in some planctomycetal genomes (Guo *et al.* 2014), we found that all the analysed species harbour essential genes for PG synthesis (Supplementary Tables 1 and 2, Supplementary Data 1-4 and Supplementary Discussion). Another main argument for the absence of a PG cell wall has been the planctomycetal resistance to beta-lactam antibiotics, which target PG biosynthesis (König *et al.* 1984). In an earlier study (Guo *et al.* 2014), an alternative cause for the observed resistance, the production of beta-lactamases, has been ruled out for Planctomycetes. However, the employed nitrocefin-based activity test for beta-lactamases is not reliable (Pitkälä *et al.* 2007) (Supplementary Figure 3 and Supplementary Note 3). Thus, we addressed the question of beta-lactam inactivating enzymes in Planctomycetes again, this time from a genomic perspective. We first identified representative proteins belonging to the beta-lactamase molecular classes A–D (Supplementary Table 3). With these sequences as queries, while employing very strict thresholds (see Supplementary Note 3 and Supplementary Discussion for details), we found at least one homologue in all the selected planctomycetal genomes (Supplementary Tables 4 and 5). Thus, Planctomycetes have the genomic potential to synthesize PG, and also to produce beta-lactamases that could confer resistance against beta-lactam antibiotics.

Biochemical Analysis of the Planctomycetal Cell Wall

In all bacteria the glycan part of PG consists of alternating residues of the β-1,4-linked amino sugars *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) (Figure 1 A; for a review see (Desmarais *et al.* 2013, Lovering *et al.* 2012)). To detect these sugar monomers, we employed a radioactive kinase assay to analyse cells of three representative planctomycetal species (*Planctomyces limnophilus*, *Gemmata obscuriglobus* and *Rhodopirellula baltica*). This assay is based on the specific MurK-mediated transfer of the radioactively labelled g-phosphoryl group from [γ-^{32}P]-ATP exclusively to the 6-position of GlcNAc and MurNAc monomers, yielding GlcNAc-6-phosphate and MurNAc-6-phosphate, respectively (Reith *et al.* 2011). Subsequent separation by thin-layer chromatography and detection via autoradiography revealed no signals for either of the sugar monomers when cells were not treated. The same result was obtained if PG was only partially digested with mutanolysin, a PG hydrolase like lysozyme, that cleaves the MurNAc-β-(1,4)-GlcNAc linkage of PG (Calandra and Cole 1980) (Figure 1 B). In contrast, after full digestion with mutanolysin, the amidases AmiD and the *N*-acetylglucosaminidase *Bs*NagZ, both sugar components were detected in all the three investigated Planctomycetes, demonstrating the presence of MurNAc and GlcNAc in polymeric glycan strands (Figure 1 B).

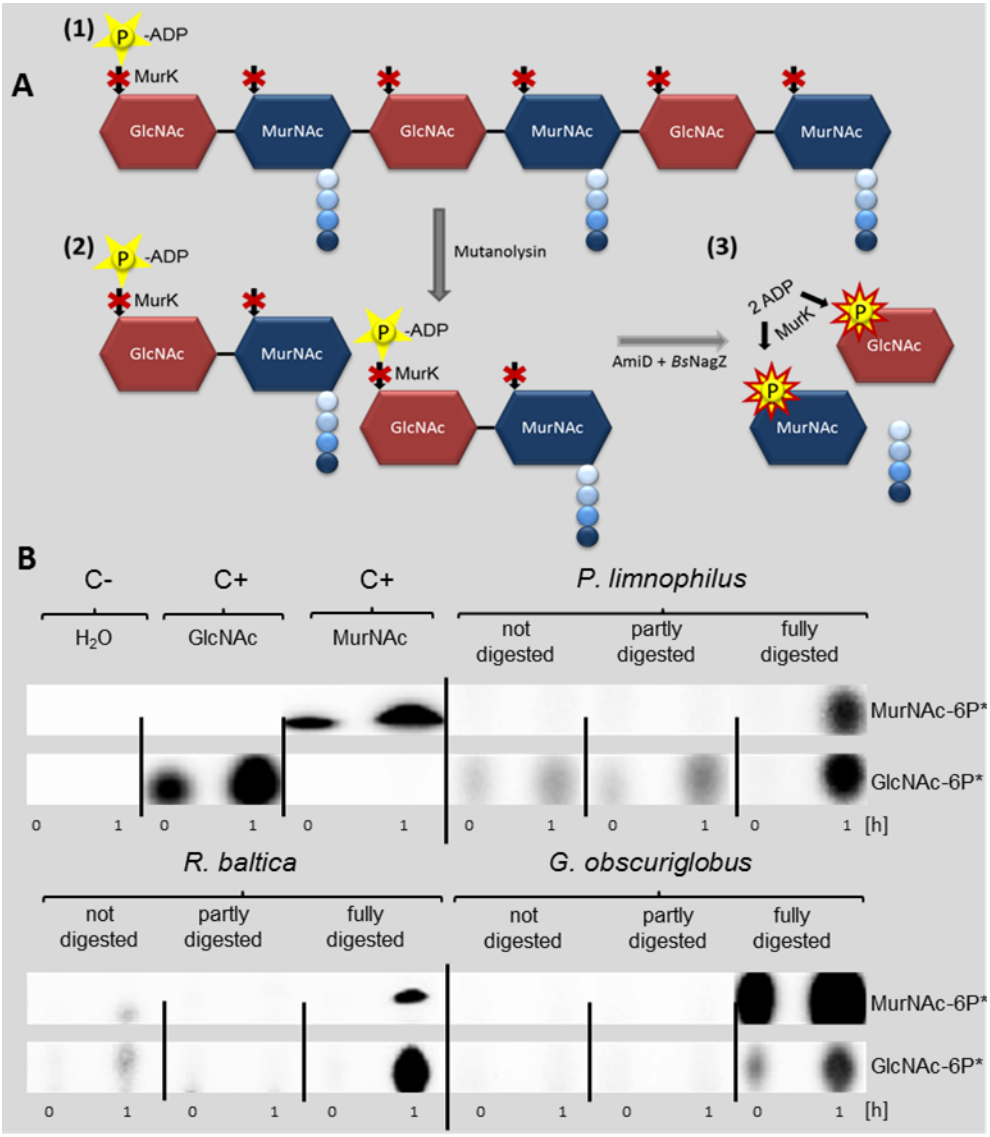

Figure 1: Radioactive labelling and detection of the PG sugars GlcNAc and MurNAc by TLC
A: Principle of the radioactive kinase assay: polymeric PG is first partly digested by mutanolysin into disaccharides (stem peptides are still linked to MurNAc). The GlcNAc/MurNAc kinase MurK is not able to transfer phosphate (P) from [γ-32P]-ATP to polymers (1) and disaccharides (2) and thus, no signal is detected in TLC. Subsequently, the disaccharides are further digested into monomers and stem peptides (3). MurK is able to transfer the [γ-32P] from [γ-32P]-ATP to the sugar monomers GlcNAc and MurNAc and thus, a radioactive signal can be detected by TLC.
B: Radioactive detection of GlcNAc and MurNAc in either undigested, partly digested (treatment with mutanolysin) or fully digested (treatment with mutanolysin, AmiD and *Bs*NagZ) preparations of *P. limnophilus*, *R. baltica* and *G. obscuriglobus* cells on incubation with [γ-32P]-ATP and MurNAc/GlcNAc kinase MurK and subsequent TLC separation. Shown are time 0 and 1 h of incubation with [γ-32P]-ATP. C^-: water negative control; C+: GlcNAc and MurNAc standards as positive controls.

In most Gram-negative bacteria these glycan strands are cross-linked via stem peptide chains by the non-proteinogenic 2,6-diaminopimelic acid (DAP), which serves as trifunctional linker, typically at the third position in stem peptides (Desmarais *et al.* 2013). The only well-documented exceptions are found among members of the Spirochaetaceae that use ornithine instead of DAP for cross-linking PG (Ben Hania *et al.* 2015, Schleifer and Joseph 1973). Consequently, the absence of DAP does not necessarily correspond to the absence of PG, while the presence of DAP is a strong indicator for cross-linked PG in bacteria. We analysed three planctomycetal model species together with strain L21-RPul-D3 for the presence of DAP by gas chromatography/mass spectrometry (GC/MS). All four investigated planctomycetal cell hydrolysates showed the specific fragment ion set for DAP at the respective retention time in the extracted ion chromatograms. The signals were significantly stronger than in *Spirochaeta asiatica* that served as a negative control (Figure 2). The observed low levels of DAP (<2.5 kCounts) in our negative controls can be explained by the de novo synthesis of lysine in which DAP is synthesized as intermediate metabolite (Schrumpf *et al.* 1991). In conclusion, our biochemical analyses provide an evidence for the existence of PG in Planctomycetes.

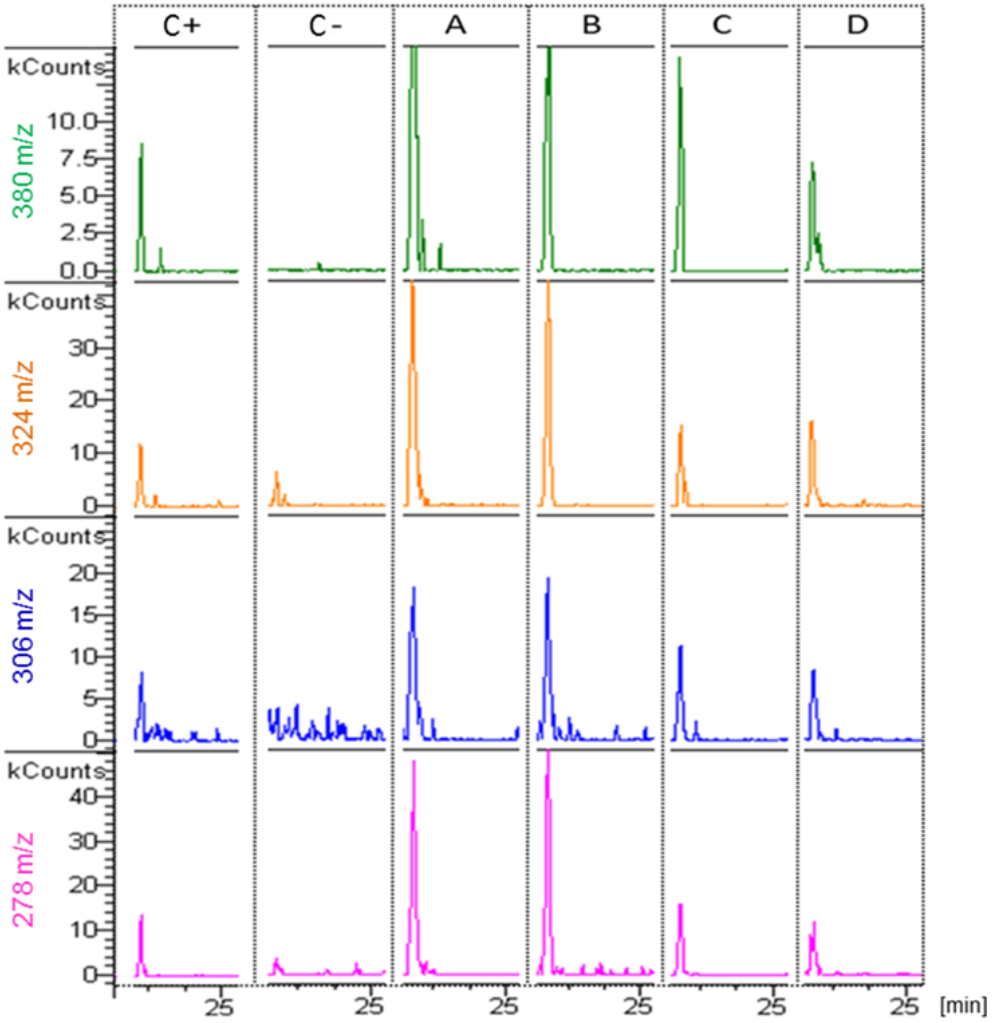

Figure 2: Mass spectrometric detection of the cell wall component (DAP) in planctomycetal cells

Ion chromatograms of the DAP derivative (N-heptafluorobutyryl 2,6-diaminopimelic acid isobutylester) from whole-cell hydrolysates of: **C+**: E. coli K12 as positive control; **C⁻**: Spirochaeta asiatica (ornithine-based PG) as negative control; **A:** P. limnophilus DSM 3776^T; **B:** G. obscuriglobus DSM 5831^T; **C:** R. baltica DSM 10527^T; **D:** L21-RPul-D3. All planctomycetal hydrolysates show peaks for specific masses (380 m/z, 324 m/z, 306 m/z, 278 m/z) and retention time (23.7 min) of DAP fragments.

Microscopic Analyses of Planctomycetal Cell Walls

Lysozyme treatment leads to cell disruption by hydrolysis of the β-1,4-linkages between MurNAc and GlcNAc28. Images of untreated Planctomycetes show subcellular details under phase contrast illumination that potentially correspond to the planctomycetal cell compartmentalization (Figure 3 A-D, panels I and II). After lysozyme treatment, the cells lose these details along with their shape or lyse entirely (Figure 3 A-D, panels III and IV). Thus, lysozyme disrupts the planctomycetal cell structure.

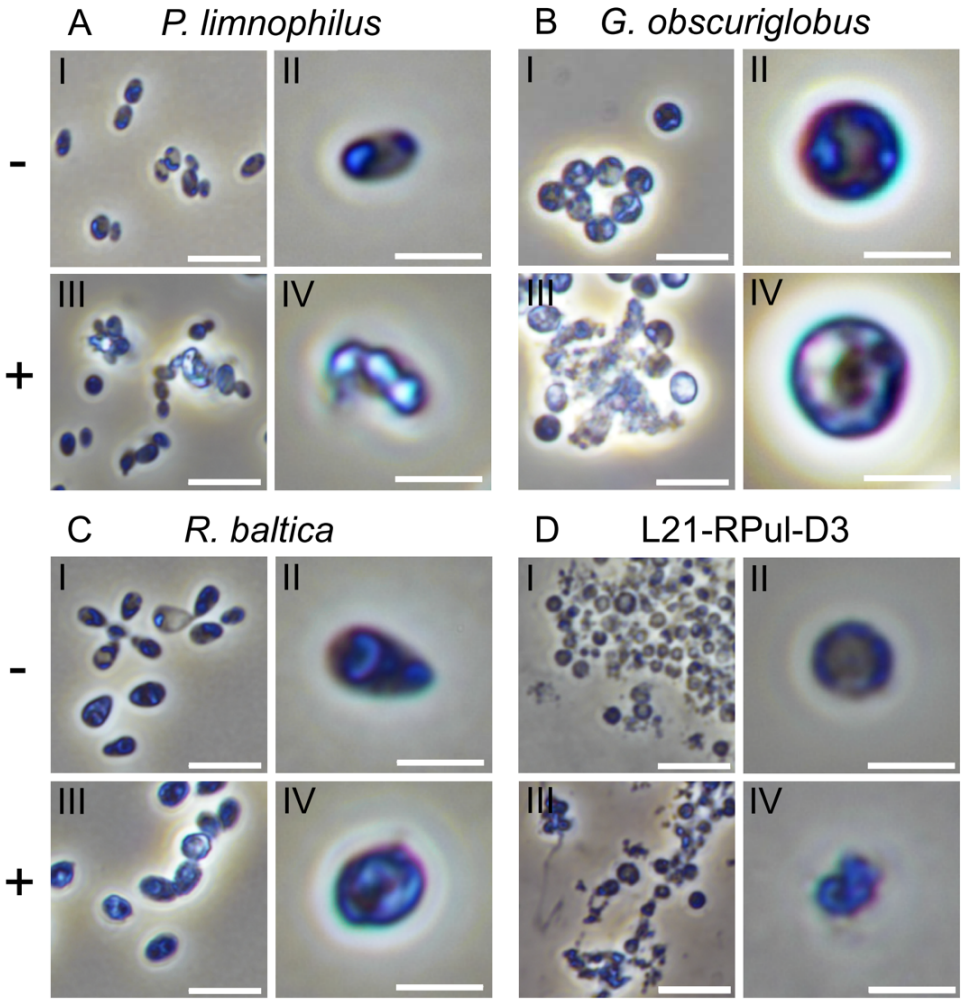

Figure 3: Effect of lysozyme on planctomycetal cells
Phase-contrast micrographs of **A:** *P. limnophilus*, **B:** *G. obscuriglobus*, **C:** *R. baltica* and **D:** L21-RPul-D3 cells after lysozyme treatment (III and IV). Untreated cells (I and II) serve as negative control. Scale bars, 5 µm (I, III) and 2 µm (II, IV).

Cell wall sacculi were prepared from *P. limnophilus* and *G. obscuriglobus* and purified from lipid and protein by treatment in a boiling SDS solution. Both species yielded intact sacculi of cell size (Figure 4 a–d) that resemble PG sacculi of other Gram-negative bacteria in shape and appearance (Yao *et al.* 1999). Treatment of isolated sacculi with lysozyme led to disintegration and fragmentation of the sacculi structures (Figure 4 e–h).

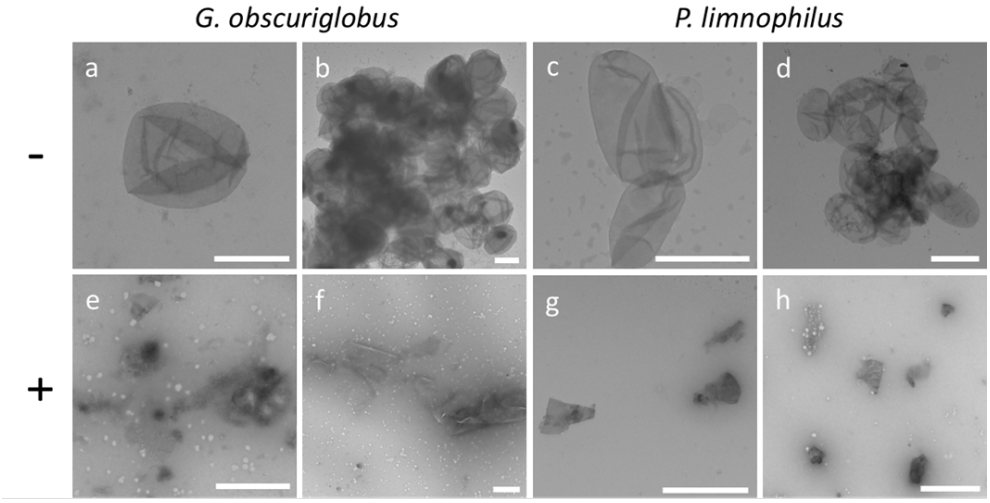

Figure 4: Effect of lysozyme on planctomycetal PG sacculi
TEM micrographs of planctomycetal cell sacculi prepared after 3 h of boiling in 10% SDS and purification through ultracentrifugation. Sacculi of *G. obscuriglobus* DSM 5831^T (**a, b, e, f**) and *P. limnophilus* DSM 3776^T (**c, d, g, h**) were untreated (**a, b, c, d**) or incubated with lysozyme (**e, f, g, h**) before negative staining with 0.1% - 2% aqueous uranyl acetate. Scale bars, 1 µm.

Moreover, we could verify the presence of DAP in *P. limnophilus* sacculi by employing our GC/MS detection method (Supplementary Figure 2). *P. limnophilus* cells vitrified and thinned by focused ion beam milling (Rigort *et al.* 2010), and investigated under near-native conditions by CET showed an electron-dense layer between the external and the internal membrane (Figure 5 and Supplementary Movie 1). This layer could be seen in cells from different growth phases and in all the 25 tomograms inspected. The location and somewhat fuzzy appearance is typical for a thin PG network of Gram-negative cells (Konorty *et al.* 2008). The optical density of the PG layer is generally poor in the polar regions and in slices that are distant from the cell's central plane (this is an effect of the oblique orientation of the PG with respect to the *x–y* slice, and of the 'missing wedge' (Konorty *et al.* 2008)). However, the cell wall layer can be detected in tomographic slices from central cell regions and particularly clearly in averaged sub frames of the cell envelope (Figure 5 B).

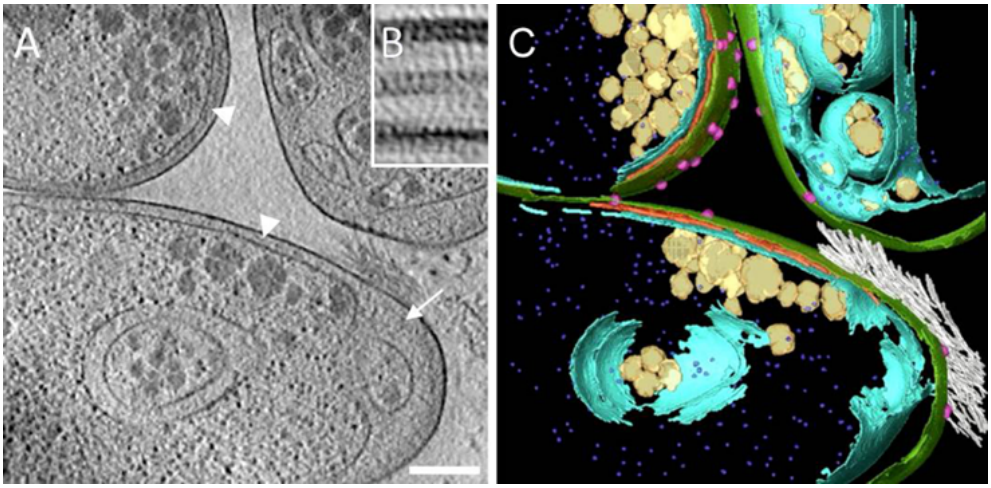

Figure 5: Cryo-electron tomography of *P. limnophilus* cells
A: Tomographic slice from a vitrified, cryo-thinned *P. limnophilus* sample showing the pole regions of three adjacent cells. A cell wall layer between the outer and inner membrane is visible in regions where the two membranes run parallel (arrowheads). The layer remains close to the outermost membrane where the inner membrane is invaginated (arrow). The reconstruction is binned three times and filtered. Scale bar, 200 nm. **B:** Average of 1,059 subframes extracted from tomographic *x*–*y* slices along the cell envelope from non-polar regions where the inner membrane (bottom) runs approximately in parallel to the outer one (top). The inner membrane is only imperfectly aligned because of its varying distance to the outer membrane (also visible in **A**). The cell wall layer in between is clearly detectable (centre-to-centre distance to the outer membrane ≈22 nm). Contrary to the inner and outer membrane layers, which show high electron density, typically originating from phosphate in lipid head groups, the PG layer produces less contrast. Size of image 45 nm x 65 nm. **C:** Segmentation of the reconstructed volume. Green: external membrane, cyan: internal membrane, red: PG layer, magenta: crateriform structures, violet: ribosomes, yellow: storage granules and white: holdfast substance

DISCUSSION

Planctomycetes belong together with Verrucomicrobia and Chlamydiae to the PVC superphylum (Wagner and Horn 2006), an unusual group of bacteria that comprise several exceptional traits (Devos *et al.* 2013). Besides Chlamydiae (Pilhofer *et al.* 2013), Planctomycetes and certain Verrucomicrobia (Yoon *et al.* 2007b) were thought to lack a PG cell wall. However, contrary to the longstanding belief, we found that Planctomycetes do possess a rigid PG cell wall. All our results are in agreement with what is known about typical constituents and features of PG from Gram-negative bacteria. Aside from specific biochemical and enzymatic evidence, we were able to isolate intact PG sacculi by harsh treatment, and could show the existence of a thin (≤ 10 nm) cell wall layer between the outer and inner planctomycetal membranes, that is the basic architecture of cell envelopes from Gram-negative bacteria. We assume that the PG sacculi completely cover the cells but can currently not exclude that local or temporary discontinuities occur. Our results put the principal hypothesis on the uniqueness of planctomycetal cellular structure to test and are in line with a number of other recent findings (Speth *et al.* 2012, Santarella-Mellwig *et al.* 2013, Devos 2014a). For instance, presence of an asymmetric outer membrane layer in Planctomycetes was suggested (Speth *et al.* 2017) and the report on the 3D structure of *G. obscuriglobus* cells (Santarella-Mellwig *et al.* 2013) challenged the current view of is compartmentalization (Fuerst and Sagulenko 2011). In this context, the existence of a PG layer, as shown in this study, is thus an important aspect as it strongly supports the 'classical' Gram-negative organization of the planctomycetal cell envelope. Whether the massive invaginations of the inner membrane observed in our tomograms of *P. limnophilus* may result in closed compartments other than a single cytoplasmic volume as recently suggested for *G. obscuriglobus* (Santarella-Mellwig *et al.* 2013) remains to be investigated in detail. Furthermore, the existence of a PG layer in Planctomycetes has implications with respect to the endocytosis-like protein uptake in Planctomycetes (Lonhienne *et al.* 2010). Vesicles of 50 to 200nm in size, which are thought to originate from the outer planctomycetal membrane by invagination (Lonhienne *et al.* 2010), could be blocked by a PG layer with a typical mesh size of 1.6–2.0 nm (Yao *et al.* 1999). Future studies have to elucidate whether the planctomycetal PG comprises pores or if the functional concept of endocytosis in Planctomycetes must be reconsidered. The results of this study mark Planctomycetes as second phylum after Chlamydiae (Pilhofer *et al.* 2013), in which simultaneous presence of PG and absence of the otherwise universal and essential bacterial cell division protein FtsZ was observed. A study on L-form *Bacillus* strains demonstrated that FtsZ became non essential in cells growing without a wall (Leaver *et al.* 2009). This study further suggested that Planctomycetes might use the same-potentially ancient-way of division (Leaver *et al.* 2009). However, our work and the lack of FtsZ among Planctomycetes (Pilhofer *et al.* 2008, Jogler *et al.* 2012) demonstrate that bacterial cell division without FtsZ does not correlate with the absence of a PG cell wall. Thus, a fundamentally different molecular mechanism of

planctomycetal cell division through budding might exist. The recent finding of PG in Chlamydiae that lack FtsZ as well (Pilhofer *et al.* 2013), further supports this interpretation and bacterial cell division might be more diverse than currently appreciated. Given that Planctomycetes (this study), anammox Planctomycetes (van Teeseling *et al.* 2015) and Chlamydiae (Pilhofer *et al.* 2013, Liechti *et al.* 2014) contain PG cell walls, the question remains whether certain Verrucomicrobia are really exceptional in this aspect (Yoon *et al.* 2007b). The methods used and developed in this study will be instrumental to address this question in the future. In conclusion our findings suggest that, despite several unique features, Planctomycetes are more Gram-negative-like than previously proposed (Fuerst and Sagulenko 2011).

ACKNOWLEDGMENT

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SUPPLEMENTARY MATERIAL

Supplementary material can be found at:
https://www.nature.com/articles/ncomms8116

SUPPLEMENTARY NOTES

https://www.nature.com/articles/ncomms8116
(PDF Files Supplementary Notes 1-3)

Supplementary Note 1. Phylogenetic Analyses

To investigate the planctomycetal cell wall composition, we first employed phylogenetic analysis to identify suitable model organisms for subsequent bioinformatic, biochemical and microscopical characterization. Since the subphylum of anammox Planctomycetes differs significantly in cell biology and metabolism from all other Planctomycetes, this group was excluded from our analysis based on previously described criteria (Jogler *et al.* 2012). Representative strains for the phylogenetic analysis were selected based on three criteria: i) type strains according to the List of Prokaryotic Names with Standing in Nomenclature ii) sequenced strains and iii) proposed type strains. When the case of several closely related strains occurred, priority was given in order of the listed criteria to represent as much diversity as possible. The phylogenetic tree in Supplementary Figure 1 contains these selected planctomycetal species including the yet unpublished deep branching, obligate anaerobic and halophilic strain L21-RPul-D3. The long branches of this tree lead to the suggestion of high evolutionary divergence of cultivated planctomycetal species. Based on phylogenetic analysis and unusual cell biological traits such as the lack of PG, Planctomycetes were hypothesized to be an exception to the bacterial definition (Fuerst and Sagulenko 2011). While our bioinformatic analysis included all sequenced planctomycetal species shown in Supplementary Figure 1 (red), biochemical experiments focused on representative organisms of each subdivision (Supplementary Figure 1, boxed in grey). Compared to the great phylogenetic depth of Planctomycetes, only few species are available in pure culture (Jogler *et al.* 2012). From those, even fewer genome sequences are available (Supplementary Figure 1, red). Thus, in total sixteen genomes were available for bioinformatics analysis.

Supplementary Note 2. Genome Mining

Comparative genomics and genome mining have been successfully employed in the past to distil a list of genes putatively involved in the unusual FtsZ-independent cell division of Planctomycetes (Jogler *et al.* 2012). In a further study, planctomycetal genome evolution was analysed. Using the metabolic pathway reconstruction of the KEGG database (Kanehisa *et al.* 2014) some, but not all PG biosynthesis related genes were identified (Guo *et al.* 2014). However, on closer inspection, this database was found to contain an almost complete set (11 of 13) of essential proteins for peptidoglycan biosynthesis in the model organism *P. limnophilus* (Jogler and Jogler 2013). Based on this observation we

propose that the distinct phylogenetic position of the PVC-superphylum and that of the Planctomycetes in particular, prevents the identification of homologous proteins by standard sequence comparison. Thus, we manually searched for PG biosynthesis related genes among sequenced Planctomycetes (Supplementary Table 2). On the other hand, a protein from *P. limnophilus* (YP_003630728.1) could be readily annotated as FlgI, the P-ring protein that anchors the bacterial flagellum in the PG layer of typical Gram-negative cells. This protein shares a significant sequence identity with FlgI from *E. coli* (WP_021570018.1: *e*-value $3e^{28}$; 31% identity). Thus, planctomycetal proteins differ in amino acid sequence conservation relative to counterparts from well-studied model organisms and a careful manual inspection is required for the identification of target proteins. In a straightforward genomic approach, one might determine whether Planctomycetes can synthesize PG from a genomic perspective by comparing all essential proteins for the synthesis of PG against the proteins encoded by the genome of sequenced Planctomycetes. However, this approach is associated with several difficulties: i) which proteins are essential for PG synthesis? ii) which query sequences of essential PG synthesis proteins should be used in comparison with Planctomycetes genes? iii) which method of comparison is appropriate? iv) how to deal with permanent draft genomes? These questions are addressed in the following.

i) Selection of essential proteins for peptidoglycan synthesis

The biosynthesis of peptidoglycan is a complex process involving approximately 20 enzymes (Lovering *et al.* 2012). Despite recent advances, the biosynthesis pathway is not completely understood yet (for review see (Bugg *et al.* 2011, Desmarais *et al.* 2013, Lovering *et al.* 2012)). Thus we focused on proteins that were previously experimentally shown to be essential for PG synthesis (Lovering *et al.* 2012) and extended our query list according to the PG pathway of the KEGG database (Kanehisa *et al.* 2014). Our analysis included the murein (PG) synthesis proteins MurA-G, the two integral membrane proteins MraY (transferase) and MviN/MurJ (flippase). In addition, we searched for penicillin binding proteins (pbp), which are targeted by beta lactam antibiotics. Apart from their role in PG formation, these proteins are also interesting as Planctomycetes are resistant against beta-lactam antibiotics. Previous studies suggested this resistance could be contributed to the lack of PG. Instead, it could be possible that the presence of beta-lactamase enzymes is responsible for the destruction of beta-lactam antibiotics (König *et al.* 1984). The entire set of proteins selected for the comparison with the database (Supplementary Data 2-4) is referred to as 'target proteins' in the following.

ii) *Influence of query sequence selection on the identification of target proteins*

In a first step, we screened for target proteins in the NCBI database employing query sequences from *E. coli*, as most of the knowledge about PG results from analysing this bacterium (Desmarais *et al.* 2013). We found that members of the genus Planctomyces encode proteins for the complete PG biosynthesis pathway, while most other Planctomycetes either lacked all or several target proteins (Supplementary Data 1). Positive results could be

verified by reciprocal BLAST analysis. In the past, others and we stopped at this point (Jogler *et al.* 2012, Pilhofer *et al.* 2008), assuming that the screening outcome concurs with the dogma that Planctomycetes lack PG (König *et al.* 1984). However, the phylogenetic distance between Enterobacteriaceae and Planctomycetes is huge. In addition, long branches in the planctomycetal phylogenetic tree (Supplementary Figure 1), which are only supported by few genomes, suggest that Planctomycetes are undersampled in sequence databases. Taken together, this could hinder protein identification based on pairwise sequence alignments. Thus, for further screening attempts, we used PG synthesis related protein sequences identified in the model organism *P. limnophilus* (Jogler *et al.* 2011) as queries. With this strategy, we found more, but still not all target proteins in the sixteen analysed genomes (Supplementary Data 2).

iii) Position-specific iterative BLAST search for target proteins

Since pairwise sequence alignments seemed to be insufficient in the quest for planctomycetal PG biosynthesis homologues we employed the PSI-BLAST algorithm at five iterations (Altschul *et al.* 1997). Using this approach, we identified most of the missing target proteins in all sequenced Planctomycetes (Supplementary Data 3). Yet, PSI-BLAST failed to detect certain target proteins that were determined with our previous strategy and verified through reverse BLAST analysis (see i) above). For example, the MurF protein from *P. brasiliensis* (YP_004270973.1) was identified employing the conventional blastp algorithm (*e*-value: $3e^{88}$; 38% identity; Supplementary Data 2). This might be caused by structurally conserved protein domains of MurC-F (Lovering *et al.* 2012). In general, except for *Planctomyces*, *Schlesneria* and *Phycisphaera*, the proteins MurC-F were identified with a rather weak *e*-value and a similarity of 13%- 31% and thus considered to be MurC-F-like proteins. However, as such proteins might be functional redundant to some degree this most likely does not affect the PG synthesis capability.

iv) Incomplete planctomycetal genomes

The genomes of *B. marina*, *G. obscuriglobus*, *Gimesia maris* (formerly known as *Planctomyces maris*, *R. maiorcia*, *R. sallentina*, *R. rubra*, *S. paludicola* and *Z. formosa* are permanent draft genomes and complete chromosome sequences are not available. Therefore, it cannot be excluded that some gene sequences were not identified because the respective draft genome assemblies did not cover the corresponding genome regions. For example, in our analysis we could not detect MurB in *R. sallentina* and MraY in *Z. formosa*.

Supplementary Note 3. Genome mining for beta-lactamase enzymes

The genome sequence of *Planctomyces limnophilus* was found to contain 13 putative beta-lactamase encoding genes (Labutti *et al.* 2010). This prompted us to employ a rigid bioinformatic screen for beta-lactamase encoding genes in the selected planctomycetal genomes (Supplementary Table 1). Through comparison with functional proteins belonging

to the molecular classes A-D and subclasses B1-B3 (selected based on (Bush 2013), Supplementary Table 3), we found planctomycetal genomes to encode between 1-19 putative beta-lactamase related proteins (Supplementary Tables 4+5). Thus at least one putative beta-lactamase gene is present in each of the analysed genomes.

Although we predicted seven and six beta-lactamase encoding genes in the genomes of *Planctomyces maris* and *Pirellula marina* (=*Blastopirellula marina*), respectively, these organisms have previously been demonstrated to lack beta-lactamase activity (Claus *et al.* 2000). However, the employed nitrocefin assay is not reliable, as it produces false negative results and fails to detect the activity in all classes of beta-lactamases (Pitkälä *et al.* 2007). Our own repetition of these experiments showed a weak beta-lactamase activity in L21-RPul-D3 cells whereas none was detected in *P. limnophilus* (Supplementary Figure 3). We predicted L21-RPul-D3 to encode one, and *P. limnophilus* to encode at least four putative beta-lactamases (Supplementary Table 4). Thus, as others concluded before, the nitrocefin assay can prove beta-lactamase activity, but a negative result does not exclude the presence of beta-lactamase enzymes. The resistance against beta-lactam antibiotics caused by a specific enzyme activity might be further supported by a 'gene dosage' effect: strain L21-RPul-D3 (one putative beta-lactamase gene, Supplementary Table 4) can withstand carbenicillin concentrations up to 100 mg/L. In contrast, *P. limnophilus* (four putative beta-lactamase genes, Supplementary Table 4) can grow with a tenfold higher concentration (1 g/L). Thus, the resistance of L21-RPul-D3 can be broken at drug concentrations, which are not yet toxic to all other Planctomycetes. In conclusion, resistance to beta-lactam antibiotics is more likely to be caused by enzymes that can be saturated rather than by a lack of PG.

SUPPLEMENTARY DISCUSSION

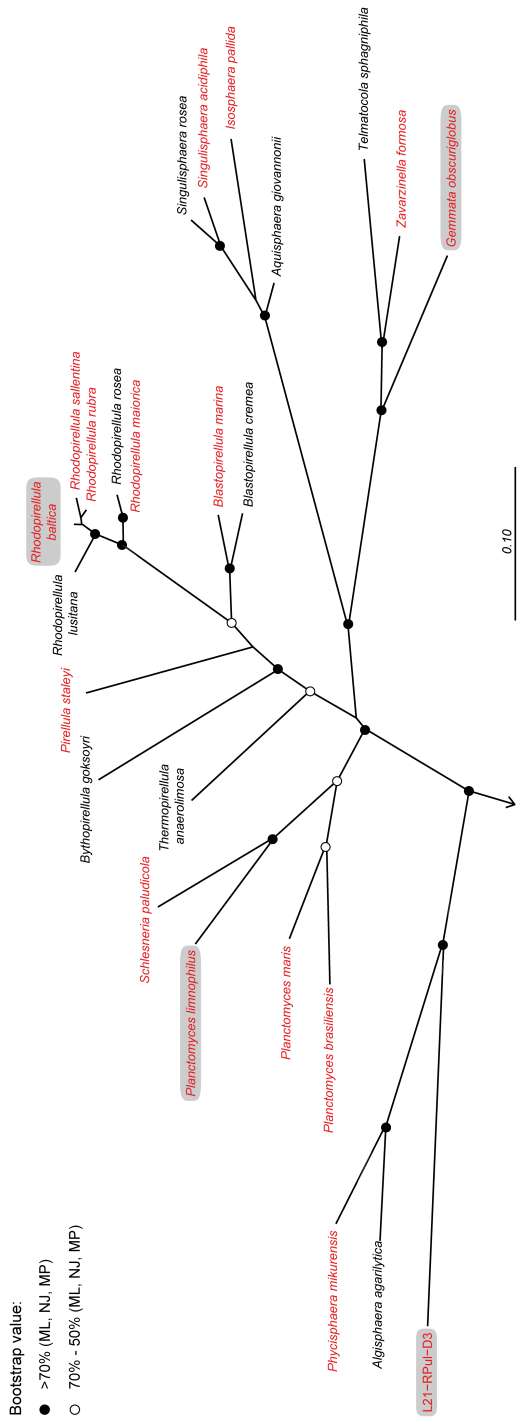
https://www.nature.com/articles/ncomms8116
(PDF Files Supplementary Discussion)

The biosynthesis of PG involves approximately 20 enzymatic reactions and is far from being entirely understood (Bugg *et al.* 2011, Lovering *et al.* 2012, Desmarais *et al.* 2013). Hence, all assumptions about a minimal list of genes required for PG production are problematic. In addition, sequences encoding the four Mur ligases (MurC-F) are known to be highly diverse among various bacterial species (15% -22% identity). On the other hand, the four proteins share three structural highly conserved domains that function in the nonribosomal peptide bond formation in PG synthesis (Bouhss *et al.* 1997). Thus, correct annotation of MurC-F with sequence based bioinformatics methods is difficult. In addition, fusion of genes involved in PG synthesis such as *murE*/*murF* or *murC*/*ddl* for example, could prevent identification (McCoy and Maurelli 2005). This might also be the case for Planctomycetes, where identification for the respective Mur ligases failed the threshold (see above). Likewise, the fact that eight of the 16 analysed planctomycetal genomes are only represented as permanent draft genomes might hinder the identification not only of the PG synthesis, but of genes in general. With the exception of the fully sequenced genome of *R. baltica* where no penicillin binding protein (pbp) and no typical flippase protein (MviN/ MurJ) could be identified, only Planctomycetes with permanent draft genomes lacked proteins for PG synthesis (see Supplementary Table 2 for summary). This explains why previous attempts could have fallen short to identify all genes required for PG synthesis in Planctomycetes (Guo *et al.* 2014). However, despite such limitations, our extended approach employing blastp combined with PSI-BLAST at 5 iterations provided evidence that, from a genomic perspective, PG production in most if not all Planctomycetes is possible. In all analysed Planctomycetes, except *G. obscuriglobus*, *I. pallida* and *Z. formosa*, MurE, the diaminopimelate ligase, was identified with an *e*-value < $6E^{-13}$ and a sequence coverage >24% (Supplementary Table 2). This indicates that Planctomycetes, as most Gram-negative bacteria, might employ diaminopimelic acid for cross-linking stem peptides in their PG sacculus. However, we were not able to identify any pbp for *R. rubra*, *R. sallentina* and *R. baltica* but succeeded for *R. maiorica*. Moreover, it appears that *R. baltica* lacks a typical flippase protein that transfers PG precursors from the cytoplasm to the periplasm. In summary, using different query sequences and blastp combined with the PSI-BLAST algorithm, we identified nearly all essential PG biosynthesis related genes in the sixteen investigated Planctomycetes (Supplementary Table 2). Our findings were confirmed employing reciprocal blastp analysis of identified putative planctomycetal PG synthesis proteins against the *E. coli* proteome (Supplementary Data 4). Thus, the bioinformatic analyses provide evidence that most, if not all Planctomycetes are able to synthesize peptidoglycan. In particular, most Planctomycetes encode pbps, which are targets for beta-lactam antibiotics. Accordingly, the resistance of all

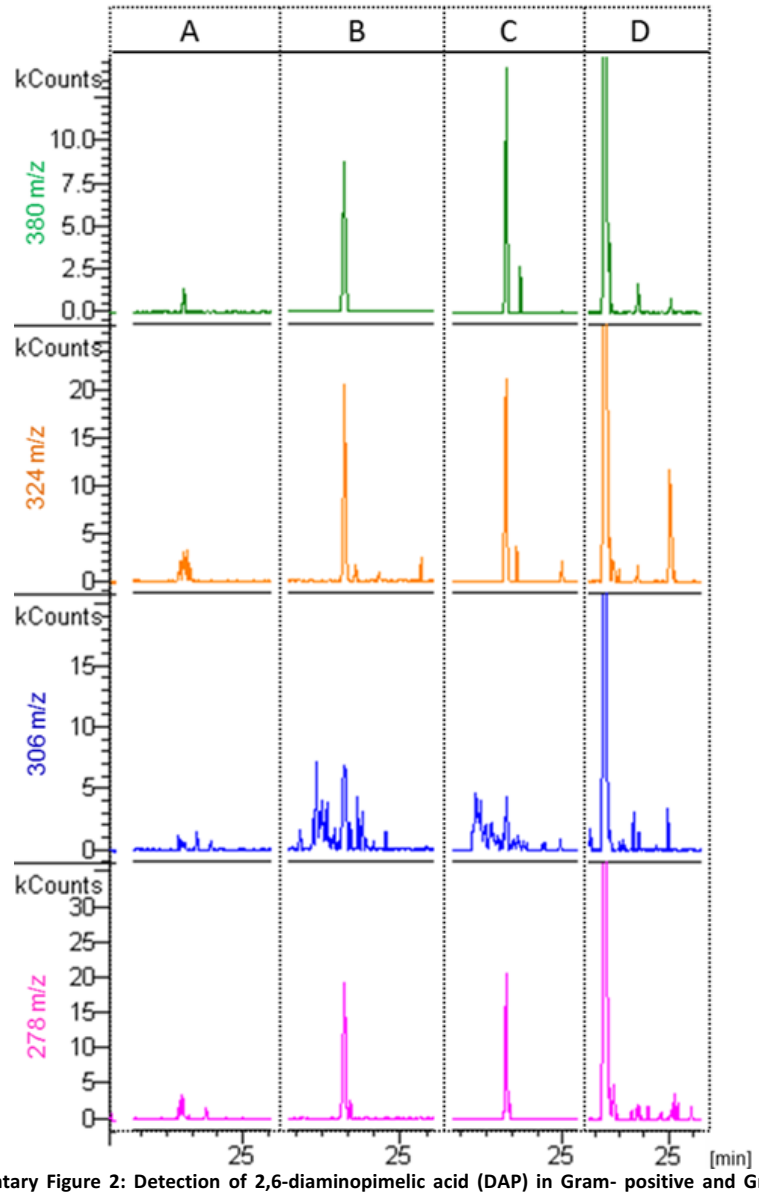
so far analysed Planctomycetes against beta-lactam antibiotics cannot be explained with the absence of PG as indicated in the literature (König *et al.* 1984).

SUPPLEMENTARY FIGURES

https://www.nature.com/articles/ncomms8116
(PDF Files Supplementary Figures 1-3)

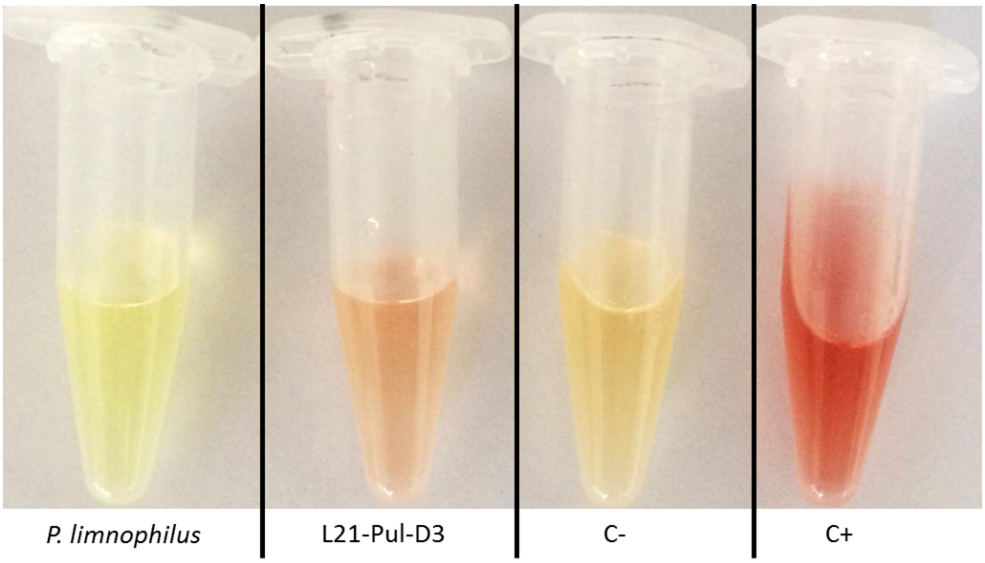


Supplementary Figure 1: Phylogenetic analysis of selected Planctomycetes
Schematic Maximum Likelihood phylogenetic tree of selected Planctomycetes based on the 16S rRNA gene sequence (for detailed information see Supplementary Table 1). Species with available genomes, used for bioinformatic analysis, are indicated in red. Species used for detailed experimental analysis in this study are highlighted in grey. Bootstrap values are based on 1000 resamplings with the Maximum Likelihood (ML), Neighbour Joining (NJ) and Maximum Parsimony (MP) algorithm. Selected anammox planctomycetes were used as out-group and are represented by an arrow ("*Candidatus* Brocadia anammoxidans", AF375994; "*Candidatus* Scalindua brodae", AY257181; "*Candidatus* Kuenenia stuttgartiensis", CT573071; "*Candidatus* Jettenia asiatica", DQ301513; "*Candidatus* Brocadia fulgida", DQ459989; "*Candidatus* Scalindua wagneri", EU478692; "*Candidatus* Anammoxoglobus propionicus", EU478694; Planctomycete KSU-1, AB057453).



Supplementary Figure 2: Detection of 2,6-diaminopimelic acid (DAP) in Gram- positive and Gram- negative bacteria and *P. limnophilus* PG sacculi.

Extracted Ion Chromatograms (EIC) of the DAP derivative (*N*-heptafluorobutyryl 2,6- diaminopimelic acid isobutylester) from murein sacculi hydrolysates. **A:** Standard cell wall mix solution (12 pmol/µL DAP) as positive control; **B:** *Catenibacterium mitsuokai* DSM 15897 as a Gram-positive control; **C:** *E. coli* DSM 498 as a Gram-negative control; **D:** *P. limnophilus* PG sacculi. The *P. limnophilus* PG sacculi hydrolysate shows the peaks for specific masses (380 m/z, 324 m/z, 306 m/z, 278 m/z) of DAP fragment ions and retention time (23.7 min) of the DAP derivative.



Supplementary Figure 3: Nitrocefin hydrolysis beta-lactamase activity assay.
Supernatant of lysed cells was treated with 50 µL nitrocefin (0.5 mg/mL). Hydrolysis of nitrocefin produces a wavelength shift from intact yellow nitrocefin (~380 nm) to degraded red nitrocefin (~500 nm). C+ positive control: *E. coli* with ampicillin resistance cassette; C- negative control: the archaeon *Methanococcus vannielii* that lacks any beta-lactamase.

SUPPLEMENTARY TABLES

https://www.nature.com/articles/ncomms8116
(PDF Files Supplementary Tables 1-5)

Supplementary Table S1: Planctomycetal reference sequences used in this study. Strains with > 99.5% 16S rRNA gene similarity to *Rhodopirellula baltica* SH1 were excluded from the analysis (*Rhodopirellula baltica* SH28, *Rhodopirellula baltica* SWK14, *Rhodopirellula baltica* WH47, *Rhodopirellula europaea* 6C, *Rhodopirellula europaea* SH398). ^{T} = type strain according to the List of Prokaryotic Names with Standing in Nomenclature (July 2014).

Species	Strain	Genome accession number	16S rRNA gene accession number
Algisphaera agarilytica	06SJR6-2	-	AB845176
Aquisphaera giovannonii	OJF2^{T}	-	NR_122081
Bythopirellula goksoyri	Pr1d	-	NR_118636
Blastopirellula cremea	LHWP2^{T}	-	NR_118153
Blastopirellula marina	SH 106^{T}	NZ_AANZ00000000	NR_029226
Gemmata obscuriglobus	UQM 2246^{T}	NZ_ABGO00000000	NR_114712
Isosphaera pallida	IS1B^{T}	NC_014962.1	NR_074534
L21−RPul−D3	L21−RPul−D3	unpublished	KC665947
Phycisphaera mikurensis	FYK2301M01^{T}	NC_017080.1	NR_074491
Pirellula staleyi	ATCC 27377^{T}	NC_013720.1	NR_074521
Planctomyces brasiliensis	DSM 5305^{T}	NC_015174.1	NR_074297
Planctomyces limnophilus	Mü 290^{T}	NC_014148.1	NR_074670
Planctomyces maris	534-30^{T}	NZ_ABCE00000000	NR_025327
Rhodopirellula baltica	SH 1^{T}	NC_005027.1	NR_043384
Rhodopirellula lusitana	UC17	-	EF589351
Rhodopirellula maiorica	SM1	ANOG00000000	FJ624363
Rhodopirellula rosea	LHWP3	-	JF748734
Rhodopirellula rubra	SWK7	ANOQ00000000	FJ624377
Rhodopirellula sallentina	SM41	ANOH00000000	FJ624360
Schlesneria paludicola	MPL7^{T}	NZ_AHZR00000000	NR_042466
Singulisphaera acidiphila	MOB10^{T}	NC_019892.1	NR_102439

Singulisphaera rosea	S26^{T}	-	NR_116969
Thermopirellula anaerolimosa	VM20-7	-	AB558583
Telmatocola sphagniphila	SP2^{T}	-	NR_118328
Zavarzinella formosa	A10^{T}	AIAB00000000	NR_042465

Supplementary Table S2: Presence or absence of predicted peptidoglycan biosynthesis proteins in planctomycetal genomes

+ : BLAST identity >30%, e-value < 1e-6, positive domain identification. In case of lower sequence identity positive domain identification was rated more important.

(+): BLAST identity >20% but <30%, e-value < $1e^{-4}$ but > $1e^{-6}$ positive domain identification.

- : No homolog detected above thresholds.

See supplementary Tables S3-S5 for details.

Protein/ Organism	MurA	MurB	MurC	MurD	MurE	MurF	MraY	MurG	MurJ/ MviN	pbp
Blastopirellula marina	+	+	(+)	(+)	+	(+)	+	+	+	+
Gemmata obscuriglobus	+	+	(+)	(+)	(+)	(+)	+	+	+	+
Isosphaera pallida	+	+	(+)	(+)	(+)	(+)	+	+	(+)	+
L21-Pul-D3	+	+	+	+	+	+	+	+	+	+
Phycisphaeara mikurensis	+	+	+	+	+	+	+	+	+	+
Pirellula staleyi	+	+	(+)	(+)	+	(+)	+	+	+	+
Planctomyces brasiliensis	+	+	+	+	+	+	+	+	+	+
Planctomyces limnophilus	+	+	+	+	+	+	+	+	+	+
lanctomyces maris	+	+	+	+	+	+	+	+	+	+
Rhodopirellula baltica SH1	+	+	(+)	(+)	+	(+)	+	+	-	-
Rhodopirellula maiorica	+	+	(+)	(+)	+	(+)	+	+	+	+
Rhodopirellula sallentina	+	-	(+)	(+)	+	(+)	+	+	(+)	-
Rhodopirellula rubra	+	+	(+)	(+)	+	+	+	+	+	-
Schlesneria paludicola	+	+	+	+	+	+	+	+	+	+
Singulisphaera acidiphila	+	+	+	(+)	+	(+)	+	+	+	+
Zavarzinella formosa	+	+	(+)	(+)	(+)	(+)	-	+	+	+

Supplementary Table 3: List of representative beta-lactam proteins belonging to the molecular classes A-D and subclasses B1-B3 used for identification of putative beta- lactamases in Planctomycetes.

Class	Species	Accession number
A	*Mycobacterium fortuitum*	Q59517
B1a	*Bacillus cereus* 569	P04190
B1b	*Klebsiella pneumoniae* 05-506	C7C422
B2	*Aeromonas hydrophila* AE036	P26918
B3	*Fluoribacter gormanii*	Q9K578
C	*Escherichia coli* K-12	P00811
D	*Acinetobacter baumannii*	Q9F0V3

Supplementary Table 4: Number of beta-lactamase related proteins found within planctomycetal genomes (details see Supplementary Table 8). The analysis is based on blastp comparison with representative proteins belonging to the molecular classes A- D and subclasses B1-B3 (Supplementary Table 5).

Species	**(Sub-) Classes[23]**						
	A	**B1a**	**B1b**	**B2**	**B3**	**C**	**D**
Blastopirellula marina	-	-	-	1	-	5	-
Gemmata obscuriglobus	1	- -	-	1	-	3	-
Isosphaera pallida	-		-	-	-	3	-
L21−RPul−D3	-	-	-	-	1	-	-
Phycisphaera mikurensis	-	-	-	-	-	1	-
Pirellula staleyi	-	-	-	-	-	4	-
Planctomyces brasiliensis	-	-	-	-	1	4	-
Planctomyces limnophilus	-	-	-	-	1	3	-
Planctomyces maris	-	-	-	1	1	5	-
Rhodopirellula baltica	-	-	-	2	-	4	-
Rhodopirellula maiorica	-	-	-	2	-	7	-
Rhodopirellula rubra	-	-	-	1	-	3	-
Rhodopirellula sallentina	-	-	-	2	-	-	-
Schlesneria paludicola	-	-	-	1	1	2	-
Singulisphaera acidiphila	1	-	-	-	2	16	-
Zavarzinella formosa	1	-	-	-	1	6	-

Supplementary Table 5: Beta-lactamase related proteins found within planctomycetal genomes based on blastp comparison with functional proteins.

Species	Accession number	Class
Blastopirellula marina	WP_002651568.1	C
	WP_002655319.1	C
	WP_002650735.1	C
	WP_002654799.1	C
	WP_002651310.1	C
	WP_002654004.1	B2
Gemmata obscuriglobus	WP_029601377.1	A
	WP_010043637.1	C
	WP_029600663.1	C
	WP_010035646.1	C
	WP_010049328.1	B2
Isosphaera pallida	WP_013564050.1	C
	WP_013566120.1	C
	WP_013566464.1	C
L21-RPul-D3	6666666.51128.peg.1253[2]	B3
Phycisphaera mikurensis	WP_014437849.1	C
Pirellula staleyi	WP_012910543.1	C
	WP_012909960.1	C
	WP_012912429.1	C
	WP_012910255.1	C
Planctomyces brasiliensis	WP_013629447.1	C
	WP_013630325.1	C
	WP_013629512.1	C
	WP_013630545.1	C
	WP_013626488.1	B3
Planctomyces limnophilus	WP_013111387.1	C
	WP_013109815.1	C

	WP_013110985.1	C
	WP_013111199.1	B3
Planctomyces maris	WP_002647322.1	C
	WP_002647812.1	C
	WP_002649364.1	C
	WP_002647636.1	C
	WP_002646057.1	C
	WP_002647025.1	B2
	WP_002648234.1	B3
Rhodopirellula baltica	WP_011118454.1	C
	WP_011121964.1	C
	WP_011123966.1	C
	WP_011118661.1	C
	WP_011123282.1	B2
	WP_011121474.1	B2
Rhodopirellula maiorica	WP_008689798.1	C
	WP_008702293.1	C
	WP_008700483.1	C
	WP_008705699.1	C
	WP_008702144.1	C
	WP_008699567.1	C
	WP_008707696.1	C
	WP_008689852.1	B2
	WP_008689849.1	B2

Rhodopirellula rubra	WP_009096753.1	C
	WP_009093330.1	C
	WP_009104091.1	C
	WP_009104526.1	B2
Rhodopirellula sallentina	WP_008674585.1	B2
	WP_008676981.1	B2
Schlesneria paludicola	WP_010586473.1	C
	WP_010584564.1	C
	WP_010581694.1	B2
	WP_010585046.1	B3
Singulisphaera acidiphila	WP_015247136.1	C
	WP_015247924.1	C
	WP_015246285.1	C
	WP_015247434.1	C
	WP_015246644.1	C
	WP_015247588.1	C
	WP_015250595.1	C
	WP_015247585.1	C
	WP_015248964.1	C
	WP_015247704.1	C
	WP_015244111.1	C
	WP_015243700.1	C
	WP_020466179.1	C
	WP_015248038.1	C
	WP_015249465.	C
	WP_015245606.1	C
	WP_015244109.1	A
	WP_015249363.1	B3
	WP_015250648.1	B3

Zavarzinella formosa	WP_020475192.1	C
	WP_020470817.1	C
	WP_020471343.1	C
	WP_020471569.1	C
	WP_020474830.1	C
	WP_020473903.1	C
	WP_020471977.1	A
	WP_020475401.1	B3

SUPPLEMENTARY DATA

Supplementary Data 1: Blastp-based analysis for the identification of peptidoglycan biosynthesis proteins in Planctomycetes employing *E. coli* proteins as query.

https://www.nature.com/articles/ncomms8116
(Excel Files Supplementary Data 1)

Essential proteins for peptidoglycan biosynthesis of *E. coli* were analysed with blastp against *P. limnophilus* proteins obtained from the NCBI database.

Supplementary Data 2: Blastp-based analysis for the identification of peptidoglycan biosynthesis proteins in Planctomycetes employing *P. limnophilus* proteins as query.

https://www.nature.com/articles/ncomms8116
(Excel Files Supplementary Data 2)

Identified PG synthesis related proteins of *P. limnophilus* (see Supplementary Data 1) were compared against all other Planctomycetes using blastp. No homologue of the penicillin-binding protein D-alanyl-D-alanine carboxypeptidase (*dac*) was found in *P. limnophilus*. Therefore, the Dac sequence from *P. brasiliensis* was used for further analyses (light green boxes) with exception for L21-RPul-D3 where the protein sequence from *P. mikurensis* was taken for BLAST analysis on the RAST server (dark green box). Proteins with a higher identity than 30%, an e-value lower than 1e-6 and with conserved domain architecture are written in light blue. Proteins below the threshold are written in red. In case of a single homologue for two different query proteins respective boxes were marked in pale red shades. In some cases no homologue was found for the respective PG biosynthesis protein (red boxes).

Supplementary Data 3: **PSI-BLAST based analysis for the identification of peptidoglycan biosynthesis proteins in Planctomycetes.**

https://www.nature.com/articles/ncomms8116
(Excel Files Supplementary Data 3)

Essential proteins for the PG biosynthesis of *E. coli* were used as query and compared against all sequenced Planctomycetes employing the PSI-BLAST algorithm. Once homologous proteins were identified in *P. limnophilus* the analysis was redone employing these planctomycetal proteins as query. No homologue to the penicillin-binding protein D-alanyl-D-alanine carboxypeptidase (Dac) was found in *P. limnophilus*. Therefore, a sequence from *P. brasiliensis* was used for further analyses (light green boxes). All homologous proteins with an identity higher than 30%, an e- value lower than 1e-6 and with conserved domain architecture, are written in light blue. Proteins identified below this threshold are written in red. In case of a single homologue for two different query proteins respective boxes were marked in pale red shades. In some cases no homologue was found for the respective peptidoglycan biosynthesis protein (red boxes). **n.a.** No sequence available at NCBI.

Supplementary Data 4: Reciprocal blastp based analysis of peptidoglycan biosynthesis proteins in Planctomycetes.

https://www.nature.com/articles/ncomms8116
(Excel Files Supplementary Data 4)

Blastp and PSI-BLAST identified PG biosynthesis related proteins (Supplementary Table 3+4) in Planctomycetes were analysed by blastp against *E. coli*. Proteins not identified using blastp or PSI-BLAST in the previous analyses (Supplementary Table 3+4) are marked with dark grey boxes. If both previous analyses resulted in the same homologous protein, the boxes are marked in light green (Supplementary Table 3+2). All homologous proteins with an identity higher than 30%, an e-value lower than 1e-6 and with conserved domain architecture, are boxed

in light blue. Homologues above threshold but with different domain architecture are written in pink. Homologues below the threshold are written in red. If no homologue was found for the respective PG biosynthesis protein in *E. coli*, boxes are marked in red.

SUPPLEMENTARY MOVIE

https://www.nature.com/articles/ncomms8116
(Videos Supplementary Movie 1)

Supplementary Movie 1
Tomographic reconstruction from a vitrified, cryo-thinned *P. limnophilus* sample showing the pole regions of three adjacent cells. A cell wall layer between the outer and inner membrane is visible in regions where the two membranes run parallel. The layer remains close to the outermost membrane where the inner membrane is invaginated. The reconstruction is binned three times and filtered. Green: external membrane, cyan: internal membrane, red: peptidoglycan layer, magenta: crateriform structures, violet: ribosomes, yellow: storage granules, white: holdfast substance.

From Genome Mining to Phenotypic Microarrays: Planctomycetes as Source for Novel Bioactive Molecules

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And was modified according to the knowledge obtained in chapter 2 and extended to planctomycetal strains Mal15^T and Pan216

ABSTRACT

Members of the phylum Planctomycetes share many unusual traits that are unique for bacteria. They divide FtsZ independent through asymmetric budding, possess a complex life cycle and comprise a compartmentalized cytoplasm. Besides their complex cell biological features Planctomycetes are environmentally important and play major roles in global matter fluxes. Such features have been successfully employed in biotechnological applications such as the anaerobic oxidation of ammonium in wastewater treatment plants or the utilization of enzymes for biotechnological processes. However, little is known about planctomycetal secondary metabolites. This is surprising as Planctomycetes have several key features in common with known producers of small bioactive molecules such as *Streptomycetes* or Myxobateria: A complex life style and large genome sizes. Planctomycetal genomes with an average size of 6.9 Mb appear as tempting targets for drug discovery approaches. To enable the hunt for bioactive molecules from Planctomycetes, we performed a comprehensive genome mining approach employing the antiSMASH secondary metabolite identification pipeline and found 102 candidate genes or clusters within the analyzed 13 genomes. However, as most genes and operons related to secondary metabolite production are exclusively expressed under certain environmental conditions, we optimized Phenotype MicroArray protocols for *Rhodopirellula baltica* and *Planctopirus limnophilia* (formerly known as *Planctomyces limnophilus*) to allow high throughput screening of putative stimulating carbon sources. Our results point towards a previously postulated relationship of Planctomycetes with algae or plants, which secret compounds that might serve as trigger to stimulate the secondary metabolite production in Planctomycetes. Thus, this study provides the necessary starting point to explore planctomycetal small molecules for drug development.

INTRODUCTION

Planctomycetes are ubiquitous bacteria that dwell in marine, freshwater and soil habitats either as free-living organisms, or attached to abiotic and biotic surfaces such as algal cells. Some representatives even live as symbionts of prawns, marine sponges or termites (Fuerst and Sagulenko 2011). Members of the Planctomycetes have been proposed to contribute substantially to the global carbon cycle (Glöckner *et al.* 2003) and to the nitrogen cycle by a unique pathway that converts ammonium to nitrogen in an oxygen independent manner (**an**aerobic **amm**onium **ox**idation, 'anammox', (Strous *et al.* 1999). Together with Verrucomicrobia and Chlamydiae, Planctomycetes form a distinct bacterial branch, the PVC-superphylum (Wagner and Horn 2006), whose members frequently exhibit a conspicuous cell plan that differs significantly from the organization of typical Gram-positive and Gram-negative bacteria (Fuerst and Webb 1991, Lindsay *et al.* 1997). Planctomycetes were proposed to lack the typical peptidoglycan layer being replaced by a proteinaceous cell wall making them resistant against beta-lactam antibiotics. This assumption was debunked in the previous chapter 2. However, it was found bacteria featuring antibiotic resistance are often in reverse producers of antibiotics. Even though, according to the current status it would seem that Planctomycetes belong to the Gram-negative bacteria (Speth *et al.* 2012, Devos 2014a, Jeske *et al.* 2015, van Teeseling *et al.* 2015) and do not possess a paryphoplasm and pirellulosome (Lindsay *et al.* 1997) they still comprise a special cell plan organization making them unique among bacteria (Boedeker *et al.* 2017). Aside from that, anammox Planctomycetes comprise indeed an additional compartment, the anammoxosome, which is confined by an unusual ladderane lipid containing membrane (van Niftrik *et al.* 2010). This compartment is the key to the anaerobic ammonium oxidation, a process of high significance for wastewater treatment that is successfully applied in industrial scale (Kartal *et al.*2010, Liu *et al.* 2008). Further, Planctomycetes and some other PVC-bacteria are unique among both bacteria and archaea as they encode proteins with a high structural similarity to eukaryotic membrane coat (MC) proteins such as clathrin. These proteins play a major role in the formation of the coated vesicles characteristic of eukaryotic endocytosis. It was proposed that in *G. obscuriglobus,* such MC-like proteins are localized in close proximity to the postulated intracytoplasmic membrane and vesicles within the paryphoplasm (Santarella-Mellwig *et al.* 2010). Moreover, vesicle-mediated protein uptake into the paryphoplasm of *G. obscuriglobus* cells, paralleling eukaryotic endocytosis, was observed (Lonhienne *et al.* 2010). Thus, *G. obscuriglobus* was postulated to possess the first vesicle-based, endocytosis-like uptake system found outside the eukaryotic domain (Jermy 2010). However, recently the presence of such vesicles was disproved by Boedeker and colleagues (Boedeker *et al.* 2017) but instead an unusual enlarged periplasm and highly curved cytoplasmic membrane were observed. Nevertheless, this novel system might play a role in the acquisition of proteins or other nutrients in natural environments. For example, Planctomycetes in biofilms on the surface of the kelp *Laminaria hyperborea* (Bengtsson and

Øvreås 2010) might take up and subsequently degrade compounds in their periplasm secreted by the kelp. Similarly, planctomycetal abundance has been correlated with a diatom bloom in the surface waters near Helgoland, a German island in the North Sea. Here 90% of the Planctomycetes were recovered in the >3 μm size fraction, indicating their attachment to diatoms (Pizzetti *et al.* 2011b). Therefore, Planctomycetes seem to be involved in the mineralization of algal biomass in aquatic habitats, which is further supported by the positive correlation of planctomycetal abundance and chlorophyll *a* concentrations in the meso-eutrophic lake, Lago di Paola, Italy (Pizzetti *et al.* 2011a). As the genome of the planctomycete *Rhodopirellula baltica* encodes more than 100 sulfatases, Planctomycetes might metabolize complex sulfated polysaccharides in such habitats (Glöckner *et al.* 2003). Some of these sulfatases display stereo- or enantioselectivity making them of great biotechnological interest (Gadler and Faber 2007, Wallner *et al.* 2005).

However, sessile cells in biofilms represent only one phase in the complex planctomycetal life cycle that also includes planktonic swimmer cells. Based on the example of *Streptomyces coelicolor* (McCormick and Flardh 2012), one would confidently predict that the spatiotemporal orchestration of planctomycetal cellular differentiation requires complex signaling cascades and regulatory networks. Indeed, it was that found planctomycetal genomes are particularly rich in extracytoplasmic function sigma factors (ECFs), with *G. obscuriglobus* being one of the most ECF-rich bacteria sequenced to date (Jogler *et al.* 2012). ECFs belong to the pool of alternative sigma factors that function as global regulators of gene expression. In addition, only a small fraction of the planctomycetal two-component regulatory systems (2CS) entail a simple one-to-one connection between a stimulus input and a single cellular response in the form of altered gene expression. Instead, the majority appears to participate in complex, interconnected, multi-step phosphate relays that might orchestrate some aspects of the complex planctomycetal life cycle. Overall, this is very reminiscent of *Myxococcus xanthus* (Jogler *et al.* 2012). In summary, Planctomycetes possess a complex cell biology and life cycle that makes them uncommon among bacteria, and their different signaling logic predicts the presence of novel bioactive signaling molecules that await discovery. In addition, Planctomycetes show resistance to several classes of antibiotics (Cayrou *et al.* 2010). Consistent with the observation that antibiotic resistance often correlates with antibiotic production, all planctomycetal genomes show a possible ability to synthesize interesting bioactive compounds. Their secondary metabolic repertoire became obvious when the first planctomycetal genome (that of *R. baltica*) had been sequenced (Glöckner *et al.* 2003). An initial prospecting in the *R. baltica* genome for genes likely to contribute to the synthesis of bioactive molecules revealed two small nonribosomal peptide synthetases (NRPSs), two monomeric polyketide synthases (PKSs), and a bimodular hybrid NRPS-PKS, all located in different chromosomal regions and thus predicted to function in the synthesis of five different unknown products. In January 2013, 13 sequenced planctomycetal genomes with an average size of 6.93 Mb were available, paving the way for the first comprehensive screen for secondary metabolite related genes in all planctomycetal

genomes within this study. Four years later not only more genomes became available but even more important also the genome mining approaches have improved significantly. Therefore, the approach was repeated with the previous organisms and was extended by including two further Planctomycetes (Mal15^T and Pan216) which are further described in the following chapters. In addition, we further investigated the planctomycetal substrate spectrum in respect to their algae/plant related lifestyle, to identify putative triggers that might stimulate the production of secondary metabolites.

MATERIAL & METHODS

Genome Mining

The online web server antiSMASH 2.0 (**anti**biotics & **S**econdary **M**etabolites **A**nalysis **SH**ell) was used for predicting secondary metabolite genes. If available, whole genome sequences were fed into the annotation and prediction pipeline as FASTA format (accession numbers see Table 1). In cases of (permanent) drafts, individual contigs were concatenated in multiple FASTA files. Before starting the antiSMASH analysis, all 24 predictable gene clusters had been chosen for analysis. The smCoG module and Gene Cluster Blast Analysis were employed for downstream applications such as gene prediction and phylogenetics as previously described (Medema *et al.* 2011). For the repeated approach the improved antiSMASH version 3.0.5 was employed (Weber *et al.* 2015). For downstream application the ClusterFinder was used with default settings (Minimum cluster size in CDS: 5, Minimum number of biosynthesis-related PFAM domains: 5, Minimum ClusterFinder probability: 60%). Furthermore, the smCoG, GeneClusterBlast, KnownClusterBlast, SubClusterBlast, ActiveSiteFinder, Detect TTA codons, Align Trans-AT PKS domains and Whole-genome PFAM analysis modules were used.

Cultivation of Selected Planctomycetes

To determine the metabolic properties of *Rhodopirellula baltica* SH1 DSM 10527^T and *Planctomyces limnophilus* DSM 3776^T we used the Phenotype MicroArray technology (Biolog, 200 Hayward, USA) (Bochner 2009). *P. limnophilus* was grown on plates solidified with 1.5% washed agar supplemented with 1 g/L peptone, 1 g/L yeast extract, 1g/L glucose, 5 mL vitamin solution (double concentrated) and 20 mL/L mineral salt solution buffered with 10 mM HEPES at pH 7.5. *R. baltica* was cultivated on plates with 1.5% washed agar supplemented with 1 g/L peptone, 1 g/L yeast extract, 1g/L glucose, 250 mL double concentrated artificial seawater (46.94 g/L NaCl, 7.84 g/L Na_2SO_4, 21.28 g/L $MgCl_2$ x $6H_2O$, 2.86 g/L $CaCl_2$ x $2H_2O$, 0.384 g/L $NaHCO_3$, 1.384 g/L KCl, 0.192 g/L KBr, 0.052 g/L H_3BO_3, 0.08 g/L $SrCl_2$ x $6H_2O$, 0.006 g/L NaF), 10 mL vitamin solution (double concentrated) and 20 mL/L mineral salt solution buffered with 5 mM Tris/HCl at pH 7.5.
Mineral salt solution and vitamin solution were prepared according to DSMZ medium 621 (http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium621.pdf). The metal salts for preparing mineral salt solution consisted of 250 mg/L Na-EDTA, 1095 mg/L $ZnSO_4 \cdot 7\ H_2O$, 500 mg/L $FeSO_4 \cdot 7H_2O$, 154 mg/L $MnSO_4 \cdot H_2O$, 39.5 $CuSO_4 \cdot 7H_2O$ mg/L, 20.3 mg/L $CoCl_2 \cdot 6H_2O$, 17.7 mg/L $Na_2B4O_7 \cdot 10H_2O$ of which 50 mL were added per liter of mineral salt solution.

Phenotype MicroArray (PM) Experiments

Agar plates of both species were grown at 28°C for three days. Subsequently, bacteria were analyzed using the Phenotype MicroArray MicroPlate PM01 and PM02 (AES Chemunex BLG 12111, BLG 12112). The inoculation media for *R. baltica* were modified according to the requirements of marine bacteria as described by Buddruhs and colleagues (Buddruhs *et al.* 2013), (for details see supplementary information). *P. limnophilus* was analyzed following the PM procedures recommended by Biolog for *B. subtilis* and other Gram-positive bacteria (Dye H), for *E. coli* and other Gram- negative bacteria (Dye A) or for fastidious Gram-negative bacteria (Dye G) (see supplementary material for details).

The cells were suspended in the inoculation medium using a sterile swab. Turbidity was adjusted to a cell density of 60% to 97% transmittance depending on the protocol used employing a turbidimeter (AES Chemunex BLG). Each well of the Phenotype MicroArray plate was inoculated with 100 µL cell suspension. The MicroPlates were sealed with parafilm, incubated at 28°C and measured in the Omnilog unit (Biolog) for 96 h. The Omnilog unit automatically reads the intensity of color formation during tetrazolium reduction every 15 min.

Data Analysis

The data obtained using the OmniLog unit (Biolog) as described above was further analyzed with the add-on package opm (Vaas *et al.* 2012, Vaas *et al.* 2013,) employing the R environment (R Development Core Team (2009) R: A Language and Environment for Statistical Computing. (R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org. ISBN 3-900051-07-0). The curve parameter A (maximum height) was determined using smoothing splines as implemented in the function do_aggr(). The maximum height values were discretized in positive, negative, and weak reactions using exact *k*-means partitioning as implemented in the function do_disc(), which is basically a wrapper for the Ckmeans.1d.dp() function (Wang and Song 2011). Distance-based k-means clustering as applied here assigns all elements in the input data into three clusters (positive, negative, weak) to minimize the sum of squares of within-cluster distances (*within*ss) from each element to its corresponding cluster center (mean) (see the help file of Ckmeans.1d.dp()). This algorithm guarantees optimal clustering; the sum of *within*ss for each cluster is always the minimum. In order to display custom curve kinetics from PM01 and PM02 plates in a single figure, the raw kinetic data of selected wells were exported from the PM01 and PM02 OPMS data objects (Vaas *et al.* 2012, Vaas *et al.* 2013) into a `flat` data frame using flatten(), merged using rbind() and then graphically displayed using the geom_path() function from the R package ggplot2 (Wickham 2009). Heatmap graphics of A values (maximum height) were obtained using the heat_map() function from the opm package under default options (Euclidian distances, Ward clustering). The Welch-Test (t-Test for data with unequal variances) was applied to each carbon source in order to test for significant differences in the A value between the two organisms. In order to keep the

family-wise error-rate (or False Discovery Rate) under control, since the Welch-Test is being calculated across 96 wells per plate, the p-values from the Welch-Test are a corrected by a Benjamini-Hochberg adjustment (Benjamini and Hochberg 1995) (Table S3).

RESULTS & DISCUSSION

Genome Mining for Secondary Metabolite Related Genes

Our genome mining approach focused on all planctomycetal genomes publically available in January 2013 (see Table S1 for details). Compared to the average bacterial genome, Planctomycetes display a rather large genome size of 6.93 Mb in average. The moderate acidophile *Singulisphaera acidiphila* (Kulichevskaya *et al.* 2008) and the stalked *Gemmata*-like planctomycete *Zavarzinella formosa* (Kulichevskaya *et al.* 2009) comprise the largest genomes (9.76 and 9.86 Mb according to NCBI respectively) (Guo *et al.* 2012), while the genome of *Phycisphaera mikurensis*, a member of the recently proposed class *Phycisphaerae*, has the smallest genome (3.88 Mb) (Fukunaga *et al.* 2009). We analyzed all genomes employing the antiSMASH annotation pipeline that allows the identification of multiple secondary metabolite related genes or gene clusters. In the first approach we found within the 13 analyzed genomes in total 102 genes or gene clusters to be putatively related with the production of secondary metabolites (Figure 2 A). Most such clusters were found in the genome of *Schlesneria paludicola* (13 clusters), closely followed by *B. marina* (twelve), *G. obscuriglobus* (twelve), *S. acidiphila* (ten) and *R. baltica* (ten). In contrast, the genomes of *P. mikurensis* and *P. KSU- 1* displayed the lowest number of clusters (two, each). This finding is not surprising, as the genome size of bacteria is known to correlate with the encoding capacity of thiotemplate modular systems (TMS) such as nonribosomal peptide synthetases (NRPS) and polyketide synthetases (PKS) which produce numerous small bioactive molecules of medical importance (Donadio *et al.* 2007). In total 44 of the 102 identified secondary metabolite related genes or clusters represent TMS. Thus, most genes were found in species with large genomes (*S. paludicola, S. acidiphila, B. marina, G. obscuriglobus* and *R. baltica*: 8.3 Mb in average), while small genomes revealed less secondary metabolite related hits (*P. mikurensis* and *P. KSU-1*: 3.9 Mb in average). However, besides TMS, planctomycetal genomes display in average a linear correlation between their size and the number of putative secondary metabolite related genes predicted by antiSMASH (Medema *et al.* 2011) (Figure 1).

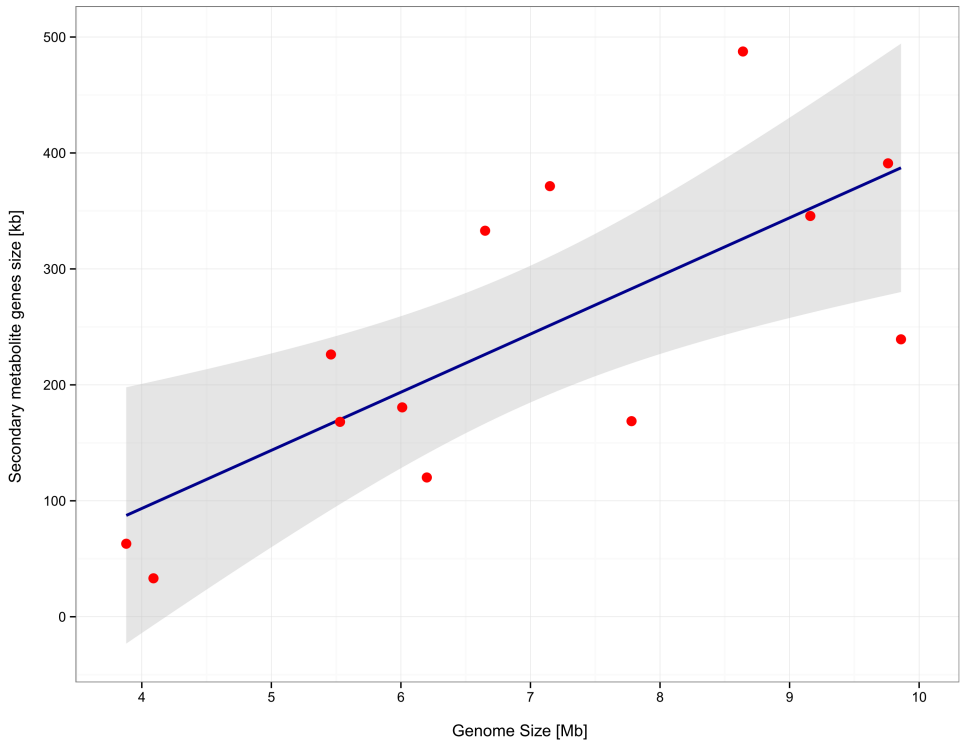

Figure 1: Correlation of genome size with secondary metabolite related genes
The genome size of all analyzed Planctomycetes (see Table S1) is plotted on the x-axis against the sum of putative secondary metabolite related gene length on the y-axis (see Figure 2 for details). Fitting a linear model on the data by using genome size as explanatory and the sum of putative secondary metabolite related gene length as response variable, more than 50% of the variance in the sum of putative secondary metabolite related gene length can be explained by the genome size (R^2 =0.54, p= 0.0041; the R function lm() was used with default parameters). The regression line based on minimizing the sum of squares of regression residuals is shown in blue. The grey shaded zone indicates the 95% confidence interval of the standard error.

In the second approach we found within the 15 analyzed genomes in total 112 genes or gene clusters to be putatively related with the production of secondary metabolites. Again most clusters were found in the genome of *Schlesneria paludicola* but twelve instead of 13 clusters. Also in the other planctomycetal genomes less cluster were identified. For instance, the genome of *B. marina* exhibits ten instead of twelve, *G. obscuriglobus* genome exhibits nine instead of twelve and likely in the genomes of *S. acidiphila* and *R. baltica* nine instead of ten cluster could be identified. The genomes of the novel strains Pan216 (7.6 Mb) and Mal15^T (9.9 Mb) revealed ten and seven secondary metabolite gene related cluster respectively. Interestingly, not only the number of clusters but also their affiliation changed. This effect might be explained by the improvement of the used algorithms. In contrast to antiSMASH version 3.0.5, employing antiSMASH version 2.0 some clusters displayed some but not all

domains of the identified secondary metabolite gene cluster. Discrimination is still not trivial especially in the case of polyketide synthases (PKS) and nonribosomal peptide synthases (NRPS), where even different types of hybrid clusters exist. A further difficulty is based on the planctomycetal genome organization. It is known for Planctomycetes that not all genes that are involved in a process are localized in an operon. For instance, in all bacteria and archaea the 16S- and 23S-rRNA along with the 5S-rRNA form an operon as they are found in clusters and are transcribed together. This is not the case for planctomycetal genomes where the 16S-rRNA seems to be localized independently from 23S- and 5S-rRNA. Therefore, the assumption of 'guilt by association' might not be applied for Planctomycetes. One possibility could be that two ore more independent identified clusters of this study might be involved in the production of one metabolite. Another assumption might be that one cluster might be involved in the modification of several products from different identified clusters. This would fit to the observation which was made regarding the ECFs in Planctomycetes. As described above only a small fraction of the planctomycetal two-component regulatory systems entail a simple one-to-one connection between a stimulus input and a single cellular response. Instead, the majority appears to participate in complex, interconnected, multi-step phosphate relays. However, the different identified genes and clusters are described as they were displayed by antiSMASH in the following section in detail (Figure 2).

Bacteriocins

Bacteriocins are antimicrobial peptides produced by certain bacteria and have recently come into focus as antibiotics. Bacteriocins show significant potency against bacteria (including antibiotic-resistant strains), are stable and display narrow to broad activity spectra (Cotter *et al.* 2013). Such compounds are structurally, functionally, and ecologically diverse and are primarily used to treat animals. However, certain bacteriocins are currently in clinical trials and it has been proposed that it is surely a question not of "if", but rather "when" these peptides will be frequently used in clinical settings (Cotter *et al.* 2013). In the first approach we found in total 17 putative bacteriocin encoding genes (Figure 2 A) and in the second only eleven (Figure 2 B) within the analyzed genomes that either showed homology to i) Cypemycin, ii) Microcin J25 or iii) Linocin M18 encoding genes. However, in the first approach seven of the putative planctomycetal bacteriocin encoding genes identified employing antiSMASH 2.0 showed homology to the domain of unknown function 692 which were excluded in the second approach. In the following all putative planctomycetal bacteriocins with a predictable function are discussed in detail:

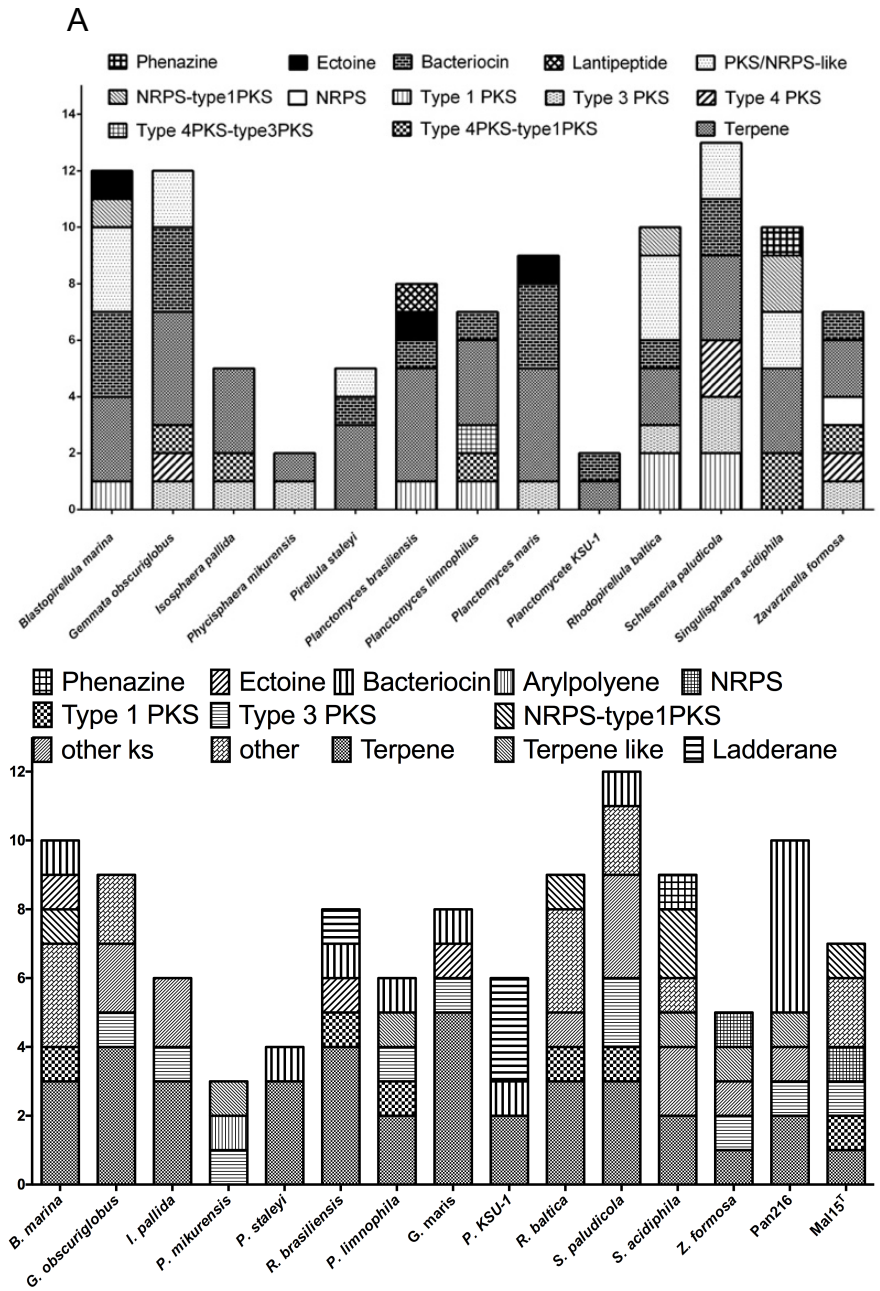

Figure 2: Planctomycetal secondary metabolite biosynthesis gene clusters

The genomes of all sequenced Planctomycetes (see Table 1 for details) were analyzed employing the antiSMASH antibiotics and secondary metabolite analysis shell. The number and type of secondary metabolite biosynthesis gene cluster is given for any genome. **A**: Results employing antiSMASH 2.0. **B**: Results employing antiSMASH 3.0.5. PKS: polyketide synthase; NRPS: nonribosomal protein synthase.

i) Cypemycin

The peptide antibiotic cypemycin was first isolated in 1993 from *Streptomyces* sp. OH-4156 and showed in vitro activity against mouse leukemia cells (Komiyama *et al.* 1993). Later the gene *cyp*A was found to encode the cypemycin precursor peptide that is subsequently extensively modified by unusual enzymes and pathways. Cypemycin established the linaridins, a class of posttranslational modified ribosomal synthesized peptides (Claesen and Bibb 2010). Two putative Cypemycin-like peptide encoding gene clusters were identified in the genomes of *B. marina* and *S. paludicola*. Such gene clusters represent interesting targets for subsequent antibiotic screening.

ii) Microcin J25

Microcin J25 belongs to the lasso-peptides, which are mainly synthesized by *Proteobacteria* and *Actinobacteria* and exhibit mostly antimicrobial activity (Rebuffat 2012). Such peptides form a structurally unique family of ribosomal synthesized natural products which are characterized by an 8- or 9- residuel macrolactam ring, formed through crosslinking of their N-terminal amino group with the side-chain carboxyl of a glutamic or aspartic-acid residue. The lasso-like arrangement is achieved by threading the C-terminal tail segment through this macrolactam ring. This entropically disfavored topology is then further stabilized by steric interactions or disulfide bond formation (Blond *et al.* 1999, Rosengren and Craik 2009). This confirmation makes microcin stable against proteases or harsh treatment (Rebuffat 2012). Microcin exerts its antimicrobial activity mimicking an iron-siderophore complex that is recognized by the outer-membrane iron transporter FhuA and subsequently transferred into the periplasm. Once transferred into the cytoplasm, microcin J25 inhibits transcription through RNA polymerase interaction (Blond *et al.* 1999, Wilson *et al.* 2003). In total, we identified seven putative microcin J25-like gene clusters in the first approach, while the genomes of *B. marina* and *Z. formosa* harbored one each. Both bacteriocins were not identified in the second approach. As well as firstly *G. obscruiglobus* was found to encode three putative microcin J25-like and none in the second approach. Similar was observed for *P. maris* (now *Gimesia maris*) since two microcin J25-likes were only identified in the first approach and only the third identiefied bacteriocine could be also found with antiSMASH 3.0.5.

iii) Linocin M18

A putative linocine M18 synthase was exclusively found in the genome of *P.* KSU-1 by both antiSMASH versions. Linocine M18 was first isolated from *Brevebacterium lines* M18 and exhibits exclusively antimicrobial activity against Gram-positives. At the time of isolation significant homology with published sequences were lacking and thus further exploration of the linocine M18-like gene cluster of *P.* KSU-1 might reveal novel insights into this class of antibiotics (Valdes- Stauber and Scherer 1994).

Lantibiotics

Lantibiotics are known to be produced by Gram-positive bacteria and show strong antimicrobial activity against a wide range of other Gram-positives. The lantibiotics are a class of more extensively modified bacteriocins and are thus discussed separately. They contain the characteristic polycyclic thioether amino acids lanthionine or methyllanthionine, as well as the unsaturated amino acids dehydroalanine and 2-aminoisobutyric acid. Like other bacteriocins they are synthesized by ribosomes, which distinguish them from most natural antibiotics, which are synthesized by specialized enzymes (Goto *et al.* 2010). Both approaches revealed one putative lantibiotic-encoding gene within the genome of *P. brasiliensis* (now *Rubinisphaera brasiliensis*) (Figure 2). As the structural and functional diversity of lantibiotics appeared to be much broader than previously imagined, they came into focus as putative therapeutics and thus the identified gene cluster is worth to be studied in detail (Willey and van der Donk 2007).

Ectoine

Ectoine (1,4,5,6-Tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) is a natural compound found in several bacterial species. Ectoines are compatible solutes, which serve as protective substances for example by acting as an osmolyte in case of extreme osmotic stress (Pastor *et al.* 2010). Consequently, it is found in high concentrations in halophilic microorganisms where it enables resistance towards salt and temperature stress. Recently, ectoines came into focus for several biotechnological applications, like macromolecule protection (*e.g.* protein stabilization) or biomedical effects such as protection against proinflammatory reactions in lung epithelial cells or antiviral activity. This increasing commercial demand started the quest for novel ectoines sources (Pastor *et al.* 2010). Planctomycetes might represent such a source as an ectoine synthesis gene cluster has been found in *B. marina* (Schwibbert *et al.* 2011). Within the two approaches we also found the already known cluster for *B. marina*. However, we identified two additional putative ectoine gene clusters within the genomes of *R. brasiliensis* and *G. maris,* which appear to be worth of further analysis (Figure 2 A).

Phenazines

Phenazines came into focus because of their anti-cancer activities. They are polycyclic aromatic compounds that contain two N-atoms within their middle ring and more than a 100 different compounds of natural origin and even more synthetic derivatives are known. Common structural features of phenazines from *Streptomyces* are isoprenylated C side chains blurring their distinction from terpenoids. This reflects the enormous chemical diversity within this group of natural products. We identified in both approaches one putative phenazine encoding gene cluster within the genome of *S. acidiphila* (Figure 2) that provides a tempting target for subsequent analysis.

Terpens

Terpens are a large and diverse class of organic compounds that are generally considered to be plant or fungal metabolites. While tens of thousands of terpenoid metabolites (modified terpens) are known, some act as antibiotics, hormones, flavor-/ odor-constituents or pigments. Others are of commercial importance because they act as vitamins or antitumor agents (Yamada *et al.* 2012). Odorous terpenoids of bacterial origin have been known for many years since the discovery of geosmin, the earth-smelling substance isolated from *Actinomycetes* being the most prominent example (Gerber and Lechevalier 1965). However, the enormous potential of prokaryotic terpenoids such as monoterpene, sesquiterpene and diterpene hydrocarbons have only recently become obvious with the advances in genome sequencing and novel bioinformatics genome mining approaches. The *in silico* prediction is a key function, as most bacterial terpenoide synthases are cryptic under normal laboratory cultivation conditions and often heterologous expression was required to identify the biochemical function of such proteins (Yamada *et al.* 2012). In addition, bacterial terpene synthases exhibit no significant overall amino acid sequence similarity to either their plant or fungal counterparts or to other synthases while some common motives exists. Therefore, conventional database searches employing the BLAST algorithm sometimes failed to identify novel bacterial terpene synthases and adapted search methods based on hidden Markov models (HMMs) for primary recognition of such enzymes were employed (Medema *et al.* 2011, Weber *et al.* 2015, Yamada *et al.* 2012). Using the antiSMASH pipeline, which is based on such HMMs, we found planctomycetal genomes to be extremely rich in terpenoid synthases gene clusters. In total we found 36 putative terpenoid synthases within the first approach in 13 genomes, while at least one of such gene clusters was present in each analyzed genome (Figure 2A) and 39 terpenoid synthases and one terpene- like gene cluster within the second approach in 15 genomes (Figure 2 B). Whereas a detailed analysis of these terpenoid synthases is beyond the scope of this study, at least some are likely to be involved in carotenoid synthesis. As most Planctomycetes form colored colonies from opal, yellow, pink or red color, carotenoid pigments are likely to be present in most, if not in all species. Preliminary chemical analysis of *P. limnophila* extracts using UV-vis and NMR spectroscopy indicate that at least three different carotinoids are present in this organism

(Jogler *et al.* unpublished observation). While carotenoid synthesis might account for several planctomycetal terpenoid synthases, others might fulfill a different function of putative interest for pharmaceutical applications and are thus worth to explore in subsequent studies. Cryptic gene clusters that confer antimicrobial activities might be stimulated through interorganismic interactions (see below) and are therefore expressed under altered ecomimetic cultivation conditions, as discussed in the Phenotype MicroArray section.

Polyketide Synthases and Nonribosomal Peptide Synthases

Polyketide synthases (PKS) and nonribosomal peptide synthases (NRPS) are multimodular enzymes that direct the formation of oligopeptide and polyketide secondary metabolites on a protein template. Both systems accomplish their task by maintaining reaction intermediates covalently bound as thioesters on the same phosphopantetheine prostetic group. In addition, each monomer consists of a separate set of enzymatic domains known as module and usually there are as many modules as monomers incorporated in the final product (Fischbach and Walsh 2006, Donadio *et al.* 2007). Such molecular assembly lines enable the production of many thousands diverse bioactive small molecules useful for various applications such as anticancer agents, antimicrobials, plant protectives or nematicides (Fischbach and Walsh 2006, Donadio *et al.* 2007, Walsh 2008). In the past years, it became evident that bacterial PKS and NRPS are much more complex than previously expected and various combinations of both assembly logics occur amongst bacteria (for detailed reviews see for example (Fischbach and Walsh 2006, Donadio *et al.* 2007, Walsh 2008)). Once the first genome of *R. baltica* became available (Glöckner *et al.* 2003), genome mining reveled two small NRPS, two modular PKS and a bimodular NRPS-PKS. Employing antiSMASH 2.0, we identified two type 1 PKS, one type 3 PKS, three PKS/NRPS-like genes and one NRPS-type 1 PKS hybrid cluster in total (Figure 2 A). In contrast, all these clusters but not the type 3 PKS were identified with the latest antiSMASH release (Figure 2 B). However, these seven or six NRPS/PKS related gene clusters include those previously identified (Donadio *et al.* 2007). The additional genes identified using antiSMASH illustrates the advances in genome mining for secondary metabolite related genes but also shows that improvement regarding Planctomycetes is necessary. Remarkably, almost all planctomycetal genomes harbored at least one NRPS or PKS related gene, only the 'small' genome of *P.* KSU-1 (4.09 Mb, Table S 1) lacked any NRPS or PKS related gene in both approaches and *P. staleyi* (6.2 Mb Tab S 1) in the second. While *S. paludicola* (genome size 8.64 Mb) containing the most (eight) (Figure 2). This again reflects the relation between the genome size and the capacity for encoding secondary metabolite related genes as demonstrated in Figure 1. Given the complexity and the unknown characteristics of PKS and NRPS produced by Planctomycetes a detailed interpretation of the identified clusters is beyond the scope of this study. However, it is noteworthy that several NRPS and PKS clusters or genes are rather small. This might point towards their role in modifying ribosomal produced peptides such as bacteriocins or their intensively modified counterparts-lantibiotics as previously reported

(Cotter *et al.* 2013). Alone the enormous amount of NRPS or PKS related genes within planctomycetal genomes makes them an interesting group of organisms to investigate the production of bioactive small molecules. However, as many NRPS and PKS clusters are not expressed under standard cultivation conditions (Wenzel and Müller 2005) alternative cultivation techniques are required to stimulate their expression. Such ecomimetic approaches call for a deeper understanding of putative trigger compounds that could enable planctomycetal expression of secondary metabolite related genes and operons. This issue will be addressed in the second part of this study.

Phenotype MicroArray Screening of Model Planctomycetes

Most secondary metabolite related genes or operons are known to be cryptic and thus only expressed under certain environmental conditions that are mostly not mimicked by axenic laboratory cultivation methods (van Wezel and McDowall 2011, Gomez-Escribano and Bibb 2012, Yamada *et al.* 2012). Consequently, most planctomycetal secondary metabolite related genes or clusters might be transcriptional silent under standard cultivation techniques. Often bioactive small molecules are produced due to environmental cues, such as inter-organismic interactions (Straight and Kolter 2009, Seyedsayamdost *et al.* 2011). Planctomycetes have been shown to be associated with eukaryotes, such as water plants or algae (Hempel *et al.* 2008, Matsuzawa *et al.* 2010, Bondoso *et al.* 2011). Consequently, we optimized a high-throughput assay based on the Phenotype MicroArray technology to deconvolute which compounds secreted by such interaction partners are recognized (metabolized) by two planctomycetal model organisms, *P. limnophilus* (limnic) and *R. baltica* (marine). This study not only provides a starting point for the analysis of other Planctomycetes, but it revealed compounds which might serve as a trigger for the activation of secondary metabolite related gene expression. We expect our findings to pave the way for an ecomimetic approach, mimicking inter organismic interactions by providing certain carbon sources and, for example, stimulating the production of antimicrobial secondary metabolites by Planctomycetes that are produced in their habitat to allow the domination of biofilms while growing on kelp (Bengtsson and Øvreås 2010, Lage and Bondoso 2011). In the following we discuss how protocols were developed to give a blueprint for the analysis of other Planctomycetes and highlight results important for modification of cultivation conditions to facilitate such an ecomimetic approach.

Phenotype MicroArray Protocol Selection

The Biolog system is based on the conversation of (carbon) substrates measured by respiration dependent NADPH production and the consequent reduction of a tetrazolium based dye. The corresponding color shift is far more sensitive than growth depending on the measured optical density (OD), as the cells only have to be able to convert the provided substrate which does not necessarily correlate with the ability to use the substrate for growth (Bochner 2009). This is of great importance, as a certain carbon substance might serve as a

trigger for the production of secondary metabolites but might not be sufficient to promote growth of Planctomycetes.

To enable phenotypic microarray screening of *R. baltica* the "PM Procedure for fastidious marine bacteria" (Dye G; Figure 3 A) and the "PM Procedure for marine bacteria" (Dye D; Figure 3 B) were evaluated employing the PM1 microarray plate (different carbon sources). With the exception of sucrose (well D11 Figure 3 A+B), both protocols had identical results. However, once experiments were repeated, these differences vanished and were never reproduced during this study. Consequently, the protocol for marine bacteria (Dye D) was selected, as previous experiments revealed a rather high background if the "PM Procedure for fastidious marine bacteria" (Dye G) was used. This might be due to the addition of the "PM additive solution" (Biolog), that includes low concentrations of additional carbon sources to increase signal to noise ratio.

For *P. limnophilus* three different protocols were evaluated employing the PM1 microarray plate. While the "PM Procedures for *B. subtilis* and other Gram-positive Bacteria" (Dye H) gave weak signals (Figure 3 C), the "PM Procedures for Gram-negative Fastidious Bacteria" (Dye G) gave optimal results (Figure 3 D). The "PM Procedures for *E. coli* and other Gram-negative bacteria" (Dye A) gave no detectable signals (data not shown). Consequently, the "PM Procedures for Gram-negative Fastidious Bacteria" (Dye G) was selected for subsequent experiments.

Negative controls without cells were conducted under identical conditions for *R. baltica* and *P. limnophilus* in order to detect spontaneous substrate conversion. However, no significant background that might result in abiotic conversion of the dye through a chemical reaction with certain carbon sources was detected.

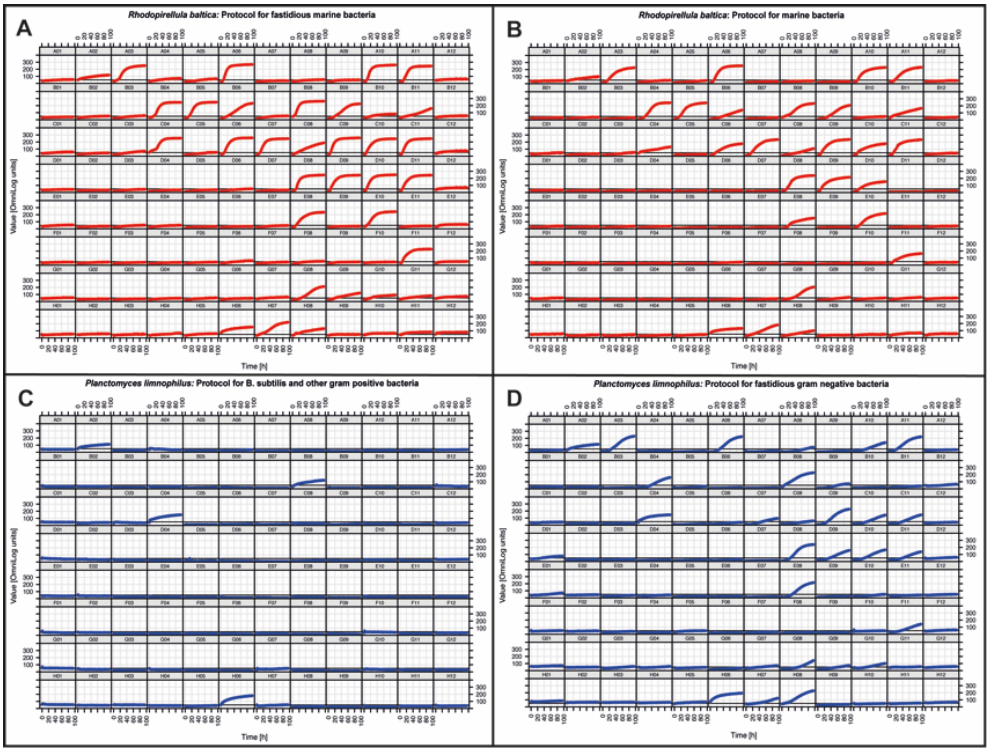

Figure 3: Phenotypic microarray protocol selection
In total five different protocols were evaluated for *Rhodopirellula baltica* (A+B, red) and *Planctomyces limnophilus* (C+D, blue) employing different carbon sources as measure (MicroPlate PM1). Curves represent dye conversion by reduction (vertical axis) during the course of time (horizontal axis). **A**: Protocol for fastidious marine bacteria **B**: Protocol for marine bacteria **C**: Protocol for *B. subtilis* and other Gram-positive bacteria. **D**: Protocol for fastidious Gram-negative bacteria. The fifth evaluated protocol (Gram-negative bacteria) gave no measurable result (data not shown).

Evaluation of Reproducibility

To evaluate the reproducibility of both protocols at least two independent experiments for plates PM1 and PM2 were performed. Cluster analysis and heat map visualization demonstrate an overall high reproducibility (Figure 4). However, if two experiments gave conflicting results, additional repetitions were performed. Figure 4 shows an exemplary comparison for plate PM2. While two replicates were sufficient for *R. baltica*, three experiments were required for *P. limnophilus*. This finding is further supported as substrate utilization patterns determined by phenotypic microarrays of both, *R. baltica* and *P. limnophilus,* are in good accordance with classical growth tests (Hirsch and Müller 1985, Schlesner *et al.* 2004) (see Table S1 A+B for details). Differences between both methods indicate a higher sensitivity of the Phenotype MicroArray technology compared to classical growth tests (Table S1 A+B). Most importantly, the phenotypic microarray hardly missed any compounds identified by classical growth rate experiments. This said, we conclude that

phenotypic microarray studies are a valid tool to analyze the spectrum of planctomycetal substrate utilization. These carbon sources can be used for 'classical' growth experiments in order to determine substrates, which can be used for cultivating in minimal medium, which is described in more detail in chapter 4.

Algae or Plants as Putative Triggers for Secondary Metabolite Production

We analyzed the phenotypic microarray results focusing on compounds produced or secreted by plants and algae. This was done according to our hypothesis that these compounds might trigger secondary metabolite production in Planctomycetes. If for example a planctomycetal cell happens to attach the surface of kelp, it might be able to take up mannitol and utilize this substrate. To protect its food source, the cell might activate expression of a bacteriocine encoding gene. The production of bacteriocines would allow the planctomycetal cell to defend its desirable habitat against other heterotrophic microbes. This hypothesis is supported by the fact that relatively slow growing Planctomycetes can dominate biofilms on kelp (Bengtsson and Øvreås 2010). Thus we propose that not necessarily the entire kelp serves as the trigger, but a secreted/produced carbon compound that could be used by the planctomycetal metabolism. Thus, such a compound might act as a starting point to create an ecomimetic cultivation broth that just by careful selection of carbon sources might simulate Kelp-Planctomycetes interaction and subsequently stimulate the production of secondary metabolites such as antibiotics. However, besides several hot candidate genes for antibiotic production, we found additional secondary metabolite biosynthesis gene (clusters) that might serve other purposes than killing. It has been shown that microbial biofilms are related with complex inter- and intra-species chemical cross-talk and the production of small molecules other than antibiotics might very well be triggered by the compounds discussed in the following (Romero *et al.* 2011).


Count
0
100
50
100
150
200
250
Value
D−Ribono−1,4−Lactone
L−Pyroglutamic Acid
L−Homoserine
L−Arginine
L−Valine
L−Histidine
N−Acetyl−L−Glutamic Acid
L−Ornithine
Acetamide
Malonic Acid
2−Hydroxy−Benzoic Acid
Quinic Acid
D,L−Octopamine
D−Citramalic Acid
L−Phenylalanine
Sebacic Acid
Citraconic Acid
Caproic Acid
L−Alaninamide
D,L−Carnitine
Glycine
Butylamine
Putrescine
N−Acetyl−D−Glucosaminitol
Sedoheptulosan
D−Lactic Acid Methyl Ester
L−Isoleucine
Capric Acid
δ−Amino−Valeric Acid
D−Arabitol
Itaconic Acid
Oxalic Acid
4−Hydroxy−Benzoic Acid
L−Lysine
D−Tartaric Acid
γ−Hydroxy−Butyric Acid
4−Hydroxy−L−Proline
Xylitol
Succinamic Acid
N−Acetyl−Neuraminic Acid
b−Cyclodextrin
g−Cyclodextrin
α−Keto−Valeric Acid
2,3−Butanediol
γ −Amino−n−Butyric Acid
m−Erythritol
L−Sorbose
β−D−Allose
Glycogen
Inulin
Gelatin
Negative Control
3−Hydroxy−2−Butanone
D−Fucose
Butyric Acid
Sorbic Acid
L−Leucine
α−Cyclodextrin
D−Tagatose
L−Methionine
2,3−Butanedione
L−Tartaric Acid
Dextrin
Chondroitin Sulfate C
β−Methyl−D−Glucuronic Acid
D−Melezitose
D−Raffinose
Laminarin
β−Hydroxy−Butyric Acid
Stachyose
Mannan
3−O−Methyl−D−Glucose
L−Arabitol
3−O−β−D−Galactopyranosyl−D−Arabinose
Pectin
Melibionic Acid
Amygdalin
L−Glucose
β−Methyl−D−Xylopyranoside
Turanose
Dihydroxy−Acetone
N−Acetyl−D−Galactosamine
α−Methyl−D−Mannoside
α−Methyl−D−Glucoside
β−Methyl−D−Galactoside
D−Lactitol
Palatinose
Maltitol
β−Gentiobiose
Arbutin
D−Salicin
2−Deoxy−D−Ribose
Oxalomalic Acid
D−Arabinose
5−Keto−D−Gluconic Acid
D−Glucosamine
Rhodopirellula baltica
Planctomyces limnophilus

Figure 4: Exemplary Phenotype MicroArray results
Heatmap display of the curve parameter A (maximum height) comparing them from *R. baltica* and *P. limnophilus* based on Phenotype MicroArrays (PM) kinetics of 95 carbon sources (microtiter plate PM02).
Visualization of the experimental data was done by employing the R-package "opm". Substrate utilization patterns of *P. limnophilus* and *R. baltica* respectively cluster according to the similarities between their metabolic activities. Substrates mentioned in the text are highlighted with yellow boxes. A dark green color indicates high metabolic activity for the corresponding substrate.

Laminarin

Laminarin is a β-1,3-linked D-glucan with occasional β-1,6-linked branches that is used for carbon storage in brown algae (Read *et al.* 1996). In contrast to *P. limnophilus*, *R. baltica* can utilize laminarin (Figure 5: red and blue curve respectively). This finding reflects the ecology of both organisms since *R. baltica* was shown to dwell on marine brown algae such as kelp, while *P. limnophilus* is adapted to freshwater habitats (Bengtsson and Øvreås 2010, Lage and Bondoso 2011).

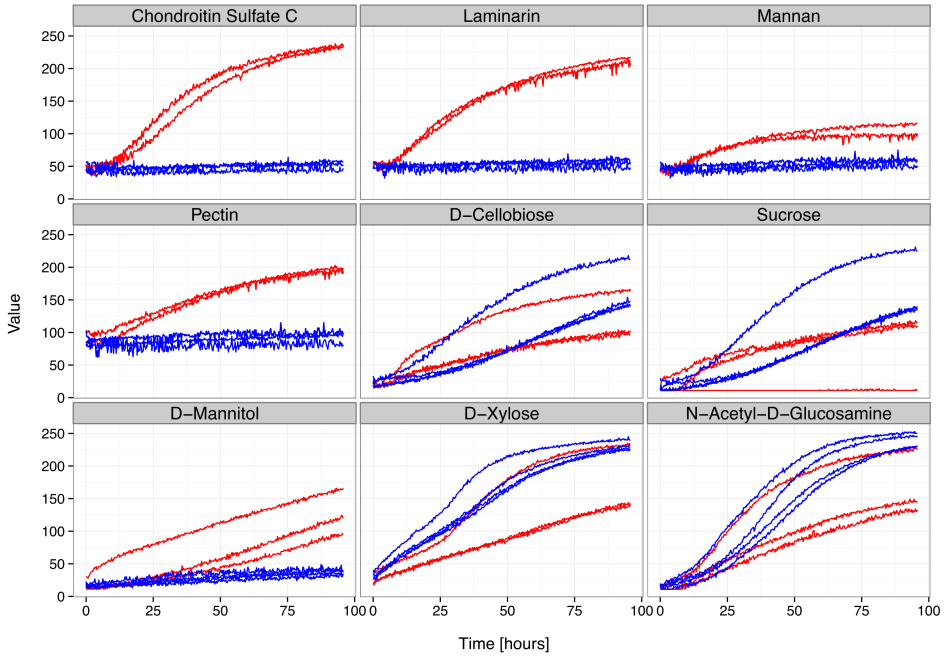

Figure 5: Phenotype MicroArray results for selected ecomimetic compounds
Phenotypic MicroArray respiration kinetics for *R. baltica* (red) and *P. limnophilus* (blue) of nine putative ecomimetic carbon substrates. Most of the substrates Planctomycetes might encounter in their native habitats and might serve as a trigger for interorganismic interactions with algae or plants. The curves show the accumulation of reduced tetrazolium dye (y-axis) over the course of time (x-axis).

Mannitol

Like laminarin, mannitol is produced by brown algae and can count for up to 30% of their dry weight (Reed *et al.* 1985, Zubia *et al.* 2008). While *R. baltica* degrades mannitol, *P. limnophilus* does not (Figure 5 B). Within marine habitats brown algae use mannitol to maintain their turgor pressure (Reed *et al.* 1985) or as storage a compound (Michel *et al.* 2010), while brown algae are rather uncommon in limnic habitats. Some *Ectocarpus* freshwater species contain only about 10% of the mannitol concentration of their marine counterparts (Dittami *et al.* 2012). Thus for *P. limnophilus* mannitol might not serve as a trigger for algae interaction as it might be absent or underrepresented within its habitat.

Mannan

The polysaccharide mannan primarily consists of D-mannose and can be found in cell walls of green- or red algae, as well as in higher plants (Popper *et al.* 2011). While *R. baltica* could moderately degrade mannan and its monomer D-mannose, *P. limnophilus* could not degrade the polymer mannan, but the monomer D-mannose (Figure 5, Table S2). However, a weak signal does not mean that mannan is less likely to be a trigger for secondary metabolite production as we measure the capability to degrade a certain carbon source for energy consumption. Weak degradation capacity could thus still mean a strong trigger signal.

Pectin

Pectin is an herbal polysaccharide that consists predominantly of α-D-galacturonic acid (Mohnen 2008) which is often methylesterified (Pelloux *et al.* 2007). Besides in higher plants, pectin can also be found in *charophyceen* green algae (Popper *et al.* 2011) and it is only degraded by *R. baltica*. Please note that in Table S3 the utilization of pectin by *P. limnophilus* is nevertheless classified by *k*-means partitioning to be 'weak', which can be explained by the constantly large (80-100) but not increasing (indicating a biologically negative reaction) OmniLog values (Figure 5). However, surprisingly, the monomer D-galacturonic acid was not utilized by *R. baltica*, while close relatives such as D-galacose, methygalactosid, methygalactoside und *N*-acetyl-D-galactosamin were metabolized.

Cellobiose

Cellobiose is a disaccharide consisting of two β-1,4-glycosidic linked glucose molecules which is part of cell walls from higher plants and algae (Popper *et al.* 2011). Cellobiose is released through cellulose degradation (Lynd *et al.* 2002) and can be used by both, *R. baltica* and *P. limnophilus* as carbon source (Figure 5). However, *R. baltica* has been shown to lack the capability to degrade cellulose before (Schlesner *et al.* 2004). One should consider that different cellulose substrates can lead to different degradation results among bacteria (Lynd *et al.* 2002), more work is needed to ultimately answer if *R. baltica* can degrade at least certain types of cellulose. In addition, under certain conditions, cellulolytic and non-cellulolytic bacteria compete against each other for the valuable substrate cellobiose that

is released by the cellulolytic species (Chen and Weimer 2001). One can envision the same situation in biofilms formed by several species on water plants and algae. Interestingly, aerobic cellulolytic bacteria are known to produce various antibiotics (Lynd *et al.* 2002). Since we identified several putative antibiotic-producing genes in Planctomycetes this might be true for cellobiose degrading organisms such as *R. baltica* as well. Thus, cellobiose might very well be a promising candidate for triggering antibiotic production in Planctomycetes.

Sucrose

Both model organisms, *R. baltica* and *P. limnophilus* show weak degradation of the disaccharide sucrose that consists of the monomers glucose and fructose, produced through photosynthetic cyanobacteria, green algae and plants (Salerno and Curatti 2003). In addition, both analyzed organisms degrade the monomers D-glucose and D-fructose.

D-Xylose

Xylose is a main component of hemicellulose that is found in all algae and higher plants (Popper *et al.* 2011). While *R. baltica* seems to degrade D-xylose at moderate rates, *P. limnophilus* gave a strong signal in our phenotypic microarray experiments. Again, the level of substrate degradation does not necessarily correlate with its function as potential trigger for secondary metabolite production.

Chondroitin Sulfate C

Chondroitin sulfates are unbranched polysaccharide chains consisting of repeating disaccharide units composed of *N*-acetylgalactosamine and glucuronic acid (Kwok *et al.* 2012), that are for example a major component of the brain matrix of higher animals (Kwok *et al.* 2012). Besides chondroitin sulfate, *R. baltica* degrades both monomers *N*-acetylgalactosamine and D-glucuronic acid, while *P. limnophilus* is only capable of *N*-acetylgalactosamine degradation (Table S2, Table S3). This again reflects the ecological niche of both organisms. While the marine *R. baltica* SH1 was shown to encode 110 sulfatases (Glöckner *et al.* 2003) and to be associated with macroalgae (Bengtsson and Øvreås 2010, Lage and Bondoso 2011), *P. limnophilus* was isolated from a freshwater habitat (Hirsch and Müller 1985). Sulfated polysaccharides are rare in limnic habitats and thus *P. limnophilus* might not encounter sufficient amounts of substrates such as chondroitin sulfate C in its natural surroundings, hence has no need to degrade them (Popper *et al.* 2011, Dantas-Santos *et al.* 2012). In contrast, marine algae possess sulfated polysaccharides within their cell wall and *R. baltica* might feed on those. Consequently, previous studies, which used 'classical' growth experiments, revealed λ-carrageenan und chondroitin sulfate degradation by *R. baltica* (Wegner *et al.* 2012). Surprisingly, no growth was observed on the sulfated polysaccharide fucoidan (Wegner *et al.* 2012). However, as only one type of fucoidan was analyzed and the chemical composition varies between different algae further investigations are required to draw a final conclusion on fucoidan utilization by *R. baltica* (Holtkamp *et al.* 2009).

N-Acetyl- D-Glucosamin

Besides macro- and microalgae, Planctomycetes can live associated with cyanobacteria (Cai *et al.* 2013). From this perspective *N*-acetyl-D-glucosamine (major component of bacterial peptidoglycan) degradation of both, *R. baltica* and *P. limnophilus* is an interesting finding. Planctomycetes might either feed on cyanobacteria or of other biofilm members on algal surfaces by degrading their cell walls. However, this hypothesis requires further investigation.

Other Utilized Di-, Tri- and Tetrasaccharides

Adjacent to the carbon substrate discussed above, both, *R. baltica* and *P. limnophilus* can degrade several di-, tri- and tetrasaccharides (see Table S2 for details). While we hypothesize that the more complex polysaccharides that are mostly more species specific might provide a better-defined clue on which surface a cell happens to attach, such compounds might serve as triggers for secondary metabolite production as well.

SUMMARY and OUTLOOK

For the first time we performed a comprehensive analysis identifying secondary metabolite biosynthesis gene clusters among all sequenced planctomycetal genomes, employing antiSMASH (Medema *et al.* 2011). This study revealed Planctomycetes as a rich source for small molecules that might ultimately lead to new antibiotics or drugs. Most secondary metabolite biosynthesis gene clusters are transcriptional silent, if standard cultivation techniques are used. Thus we adjusted screening protocols based on the Phenotype MicroArray technology for carbon sources that are recognized and degraded by two model organisms, *R. baltica* and *P. limnophilus*. We identified various compounds; mostly polysaccharides that are known to be plant or algae related and that might serve as a trigger for secondary metabolite production. Consequently, our study provides a starting point for the analysis and biotechnological application of small molecules of planctomycetal origin.

ACKNOWLEDGMENT

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SUPPLEMENTARY MATERIAL

Supplementary material can be found at:
https://link.springer.com/article/10.1007/s10482-013-0007-1

Supplementary Information

https://link.springer.com/article/10.1007/s10482-013-0007-1
(10482_2013_7_MOESM1_ESM.pdf)

PM Procedures
All procedures were tested with the PM 1 plate, only for the optimal protocols also PM 2 were tested.

PM Procedures for marine Bacteria (Buddruhs *et al.* 2013)

PM 1/PM 2:

10 mL	1.2x IF-0 (AES Chemunex BLG 72268)
1200 µL	10x artificial seawater stock solution [1]
120 µL	100x vitamin stock solution [2]
12 µL	1000x trace element stock solution[3]
120 µL	100x $NaHCO_3$ Buffer (19 g/L)
120 µL	Dye D (AES Chemunex BLG 74224)
428 µL	sterile H_2O

Turbidity: 80-85% of *Rhodopirellula baltica* cells

PM Procedures for fastidious marine bacteria (unpublished)

PM 1:

10 mL	1.2x IF-0 (AES Chemunex BLG 72268)
1.2 mL	10x artificial seawater stock solution [1]
120 µL	100x vitamin stock solution [2]
12 µL	1000x trace element stock solution[3]
120 µL	100x $NaHCO_3$ Buffer (19 g/L)
120 µL	Dye G (AES Chemunex BLG 74227)
428 µL	12x PM Additive Solution[4]

Turbidity: 60% of *Rhodopirellula baltica* cells

PM Procedures for Gram-negative fastidious bacteria (as recommended by Biolog)

PM 1/PM 2:

10 mL	1.2x IF-0 (AES Chemunex BLG 72268)
120 µL	Dye G (AES Chemunex BLG 74227)
1 mL	12x PM Additive Solution[4]
880 µL	*P. limnophilus* cells with a turbidity of 5% in IF-0 medium

Turbidity: 65% of *Planctomyces limnophilus* cells

PM Procedures for *B. subtilis* and other Gram-positive bacteria (as recommended by Biolog)

PM1:

10 mL	1.2x IF-0 (AES Chemunex BLG 72268)
120 µL	Dye H (AES Chemunex BLG 74228)
1 mL	12x PM Additive Solution[5]
880 µL	sterile H_2O

Turbidity: 97% of *Planctomyces limnophilus* cells

PM Procedures for *E. coli* and other Gram-negative bacteria (as recommended by Biolog)

PM1:

10 mL	1.2x IF-0 (AES Chemunex BLG 72268)
120 µL	Dye A (AES Chemunex BLG 74221)
1880 µL	sterile H_2O

Turbidity: 85% of *Planctomyces limnophilus* cells

[1] 10x artificial seawater stock solution: 200 g NaCl, 40 g Na_2SO_4, 30 g $MgCl_2 \cdot 6\ H_2O$, 5 g KCl, 2.5 g NH_4Cl, 2 g KH_2PO_4, 1.5 g $CaCl_2 \cdot 2\ H_2O$

[2] 100x vitamin stock solution: 100 mg thiamine, 20 mg niacin, 8 mg 4-aminobenzoic acid, 2 mg biotin.

[3] 1000x trace element stock solution: 2.1 g $FeSO_4 \cdot 7\ H_2O$, 13 mL 25% HCl, 5.2 g Titriplex III (Na_2EDTA; adjust pH to 6.0-6.5 to resolve), 190 mg $CoCl_2 \cdot 6\ H_2O$, 144 mg $ZnSO_4 \cdot 7\ H_2O$, 100 mg $MnCl_2 \cdot 4\ H_20$, 36 mg $Na_2MoO_4 \cdot 2\ H_2O$, 30 mg H_3BO_3, 24 mg $NiCl_2 \cdot 6\ H_2O$, 2 mg $CuCl_2 \cdot 2\ H_2O$.

[4] 12x PM Additive Solution for Gram-negative fastidious bacteria (PM 1 and PM 2), also used for fastidious marine bacteria (PM1 and PM 2): 24 mM $MgCl_2 \cdot 6H_2O$, 12 mM $CaCl_2 \cdot 2\ H_2O$, 0.3 mM L-arginine (HCl), 0.6 mM Sodium L-glutamic acid, 0.3 hypoxanthine, 0.06 mM Sodium β-NAD, 0.3 mM 5'-UMP (disodium salt), 0.3 mM L-cysteine (pH 8.5), 0.06% yeast extract, 0.06% tween 80.

[5] 12x PM Additive Solution for *B. subtilis* and other Gram-positive bacteria (PM 1 and PM 2): 24 mM $MgCl_2 \cdot 6\ H_2O$, 12 mM $CaCl_2 \cdot 2H_2O$, 0.15 mM L-cysteine (pH 8.5), 0.3 mM 5'-UMP (disodium salt), 0.3 mM L-arginine (HCl), 0.6 mM L-glutamic acid, 0.06% yeast extract, 0.06% tween 80.

SUPPLEMENTARY TABLES

Supplementary Table S1: Characteristics of sequenced planctomycetal genomes

https://link.springer.com/article/10.1007/s10482-013-0007-1
(10482_2013_7_MOESM2_ESM.pdf)

Clusters refers to the number of predicted secondary metabolite related genes or cluster with the individual genomes(seeTableS2)

Organism	Chromosome	Cluster	Sequenze	Plasmid	Genes	Protein	Type strain	Accession numbers
Blastopirellula marina	6.65MB	12	draft	-	6079	6025	DSM 3645	NZ_AANZ00000000
Gemmata obscuriglobus	9.16MB	12	draft	-	8080	7989	UQM 2246	NZ_ABGO00000000
Isosphaera pallida[1]	5.53MB	5	complete	1	3823	3722	ATCC 43644	NC_014962.1
Phycisphaera mikurensis	3.88MB	2	complete	1	3334	3282	NBRC 102666	NC_017080.1
Pirellula staleyi[2]	6.2MB	5	complete	-	4825	4717	DSM 6068	NC_013720.1
Planctomyces brasiliensis	6.01MB	8	complete	-	4865	4750	DSM 5305	NC_015174.1
Planctomyces limnophilus[3]	5.46MB	7	complete	1	4373	4258	DSM 3776	NC_014148.1
Planctomyces maris	7.78MB	9	draft	-	6530	6480	DSM 8797	NZ_ABCE00000000
Planctomycete KSU-1[4]	4.09MB	2	draft	-	3653	3600	n.a.	NZ_BAFH00000000
Rhodopirellula baltica[5]	7.15MB	10	complete	-	7404	7325	DSM 10527	NC_005027.1
Schlesneria paludicola[6]	8.64MB	13	draft	-	7080	6998	DSM 18645	NZ_AHZR00000000
Singulisphaera acidiphila[6]	9.76MB	10	complete	3	7683	7251	DSM 18658	NC_019892.1
Zavarzinella formosa	9.86 MB	7	draft	-	n.a.	n.a.	DSM 19928	AIAB00000000

Supplementary Table S2: This table shows all identified gene clusters employing

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(10482_2013_7_MOESM3_ESM.pdf)

antiSMASH. Each gene was compared against the NCBI database using BlastP (proteinprotein BLAST). In the left column one finds the locus tag or, alternatively in case of permanent draft genomes, the corresponding protein accession. In the right column a brief description of the identified gene is given. The color code and the order correspond to the antiSMASH prediction and are identical in the table and genemap of each section. Red highlighted genes and rows show biosynthetic, blue highlighted show transport-related, green highlighted show regulatory and grey or white show other genes and proteins. If the blasting result differed from the color code used by antiSMASH the antiSMASH color code was kept. Proteins, which accession numbers are highlighted in red or dark blue showed *e*-values higher than $1e^{-10}$ for the protein in the corresponding right row.

Supplementary Table S3: Phenotype MicroArray results

https://link.springer.com/article/10.1007/s10482-013-0007-1
(10482_2013_7_MOESM4_ESM.pdf)

Supplementary Table S3 A: Phenotype MicroArray results for *Rhodopirellula baltica* compared to results of classical growth tests after Schlesner et al. 2004. If known, the usage of carbon sources L- or D- forms are indicated (+, positive; -, negative; w, weak signal).

Carbon source	*R. baltica* (Biolog)	*R. baltica* (growth test)
N-acetylglucosamine	w	+
adonitol	-	-
alanine	- (L) – (D)	-
amygdalin	+	+
arabitol	w (L) - (D)	-
arginine	- (L)	-
asparagine	- (L)	-
cellobiose	w (D)	+
chondroitin sulfate	+	+
dextrin	+	+
dulcitol	-	-
formate	-	-
fructose	+ (D)	+
fucose	w (L) – (D)	-
galactose	+ (D)	+
glucose	w (D)	+
glutamine	- (L)	-
glycerol	-	+
glycine	-	-
histidine	- (L)	-
leucine	- (L)	-
inulin	-	-
isoleucine	-L	-
lactose	w (D)	+
lysine	- (L)	-
lyxose	w (L)	+
maltose	w (D)	+
mannitol	w (D)	-
mannose	+ (D)	+
melezitose	+ (D)	+

melibiose	+ (D)	+
methionine,	w/- (L)	-
ornithine	- (L)	-
pectin	+	-
phenylalanine	- (L)	-

Supplementary Table S3 B: Phenotype MicroArray results for *Planctomyces limnophilus* compared to results of classical growth tests after Hirsch and Müller 1985. If known, the usage of carbon sources L- or D- forms are indicated (+: positive; --: negative; w: weak signal).

Carbon source	***P. limnophilus* (Biolog)**	***P. limnophilus* (growth test)**
N-acetylglucosamine	+	+
alanine	- (L) – (D)	- (L) – (D)
arginine	- (L)	- (L)
cellobiose	w (D)	+
dextrin	-	-
formate	-	-
fructose	+/w (D)	- (D)
galactose	+ (D)	+ (D)
glucuronic acid	- (D)	-
glycerol	-	-
glycine	-	- (L)
histidine	- (L)	- (L)
leucine	- (L)	- (L)
inulin	-	-
lactose	w (D)	+ (D)
mannitol	-	-
phenylalanine	- (L)	- (L) - (D)
proline	- (L)	- (L)
ribose	w (D)	- (D)
salicin	+ (D)	-
serine	- (L) – (D)	- (L)

Supplementary Table S4

https://link.springer.com/article/10.1007/s10482-013-0007-1
(10482_2013_7_MOESM5_ESM.pdf)
Carbon sources that were converted at least by one of the tested planctomycetal species. -: negative reaction; w: weak reaction; +: positive reaction.

Carbon Source	*R. baltica*	*P. limnophilus*
Acetic Acid	w	-
N-acetyl-D-Galactosamine	+	+
N-acetyl-D-Glucosamine	w	+
N-acetyl-β-D-Mannosamine	+	w
β-D-Allose	w/-	-
Amygdalin	+	-
D-Arabinose	w	w
L-Arabinose	w	w
L-Arabitol	w	-
Arbutin	w/-	+
Butyric Acid	w/-	-
D-Cellobiose	w	w
Chondroitin Sulfate C	+	-
2-Deoxy-D-Ribose	w	w
Dextrin	+	-
Dihydroxy-Acetone	+	+
D-Fructose	+	w/-
D-Fucose	-	w
L-Fucose	w	w
3-O-β-D-Galactopyranosyl- D-Arabinose	+/w	-

D-Galactose	+	+
Gelatin	w	-
β-Gentiobiose	+	w
D-Gluconic Acid	w	-
D-Glucosamine	w	w
D-Glucose	w	+
L-Glucose	+/w	-
Glucuronamide	w	w
D-Glucuronic Acid	+	-
3-Hydroxy-2-Butanone	w/-	-
5-Keto-D-Gluconic Acid	w	w
L-Lactic Acid	+	-
D-Lactitol	+	-/w/+
α-D-Lactose	w	w
Lactulose	w	w
Laminarin	+	-
L-Lyxose	w	+
D-Maltose	w	w
Maltotriose	w	-
Maltitol	+	w
Mannan	w	-
D-Mannitol	w	-
D-Mannose	+	+
D-Melezitose	+	-
Melibionic Acid	w/+	-

D-Melibiose	+	w
L-Methionine	w/-	-
α-Methyl-D-Galactoside	+	+
β-Methyl-D-Galactoside	+	+
3-O-Methyl-D-Glucose	w	-
α-Methyl-D-Glucoside	+	+
β-Methyl-D-Glucoside	w	+
β-Methyl-D-Glucuronic Acid	+	-
α-Methyl-D-Mannoside	+	+
Methyl Pyruvate	-	w
β-Methyl-D-Xylopyranoside	+	-
Oxalomalic Acid	w	w
Palatinose	+	w
Pectin	+	w
Pyruvic Acid	w	+
D-Raffinose	+	-
D-Ribose	w	w
D-Salicin	w	+
Sorbic Acid	w	-
L-Sorbose	w/-	-
Stachyose	w	-
Sucrose	w	w
D-Trehalose	w	w
Turanose	+	+
D-Xylose	w	+

Supplementary Table S5:

https://link.springer.com/article/10.1007/s10482-013-0007-1
(10482_2013_7_MOESM6_ESM.pdf)

Carbon sources that differ regarding the metabolic conversation of *R. baltica* and *P. limnophilus.* (-: negative reaction; w: weak reaction; +: positive reaction) Carbon sources that differ significantly applying the Welch test were indicated with "x".

Carbon Source	***Rhodopirellula baltica***	***Planctomyces limnophilus***	**Welch-Test (p<0.05)**	**Welch-Test (p<0.05) with BH adjustment**
Acetic Acid	w	-	x	x
N-acetyl-D-Glucosamine	w	+		
N-acetyl-β-D-Mannosamine	+	w		
β-D-Allose	w/-	-	x	
Amygdalin	+	-	x	
L-Arabitol	w	-		
Arbutin	w/-	+		
Butyric Acid	w/-	-		
Chondroitin Sulfate C	+	-	x	x
Dextrin	+	-	x	x
D-Fructose	+	w/-	x	
D-Fucose	-	w		
3-O-β-D-Galactopyranosyl-D- Arabinose	+/w	-		
Gelatin	w	-	x	
β-Gentiobiose	+	w		
D-Gluconic Acid	w	-	x	
D-Glucose	w	+		
L-Glucose	+/w	-		
D-Glucuronic Acid	+	-	x	x
3-Hydroxy-2-Butanone	w/-	-		

L-Lactic Acid	+	-	x	
D-Lactitol	+	-/w/+		
Laminarin	+	-	x	x
L-Lyxose	w	+	x	x
Maltotriose	w	-	x	
Maltitol	+	w		
Mannan	w	-		
D-Mannitol	w	-	x	
D-Melezitose	+	-	x	

Developing Techniques for the Utilization of Planctomycetes as Producers of Bioactive Molecules

This chapter has been published as:

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ABSTRACT

Planctomycetes are conspicuous, ubiquitous, environmentally important bacteria. They can attach to various surfaces in aquatic habitats and form biofilms. Their unique FtsZ-independent budding cell division mechanism is associated with slow growth and doubling times from six hours up to one month. Despite this putative disadvantage in the struggle to colonize surfaces, Planctomycetes are frequently associated with aquatic phototrophic organisms such as diatoms, cyanobacteria or kelp, whereby Planctomycetes can account for up to 50% of the biofilm-forming bacterial population. Consequently, Planctomycetes were postulated to play an important role in carbon utilization, for example as scavengers after phototrophic blooms. However, given their observed slow growth, such findings are surprising since other faster- growing heterotrophs tend to colonize similar ecological niches. Accordingly, Planctomycetes were suspected to produce antibiotics for habitat protection in response to the attachment on phototrophs. Recently, we demonstrated their genomic potential to produce nonribosomal peptides, polyketides, bacteriocins, and terpenoids that might have antibiotic activities. In this study, we describe the development of a pipeline that consists of tools and procedures to cultivate Planctomycetes for the production of antimicrobial compounds in a chemically- defined medium and a procedure to chemically mimic their interaction with bacteria such as for example Cyanobacteria. We evaluated and adjusted screening assays to allow the hunt for planctomycetal antibiotics. As proof of principle, we demonstrate antimicrobial activities of planctomycetal extracts from *Planctopirus limnophila* DSM 3776, *Rhodopirellula baltica* DSM 10527, and the recently isolated strain Pan216. By combining UV/Vis and high resolution mass spectrometry data from High-Performance Liquid Chromatography fractionations with growth inhibition of indicator strains, we were able to assign the antibiotic activity to candidate peaks related to planctomycetal antimicrobial compounds. The MS analysis points towards the production of novel bioactive molecules with novel structures. Consequently, we developed a large scale cultivation procedure to allow future structural elucidation of such compounds.

Our findings might have implications for the discovery of novel antibiotics as Planctomycetes represent a yet untapped resource that could be developed by employing the tools and methods described in this study.

INTRODUCTION

Antibiotics are mostly small molecules with antimicrobial activity produced by nearly all sorts of living things. They revolutionized treatment of infectious diseases, saving countless lives. The ability to produce such active molecules -mostly as secondary metabolites- is unevenly distributed among different species (Berdy 2005). In the kingdom of bacteria, Actinomycetes are the best studied producers, followed by Myxobacteria, Cyanobacteria and certain *Bacillus* and *Pseudomonas* strains. In total about 13,800 'active' compounds of bacterial origin are known (Berdy 2005). However, these 'usual suspects' in terms of antibiotic production have been heavily screened in the past and the discovery of novel lead structures decreased, while rediscovery rates of known compounds increased (Cooper and Shlaes 2011). Consequently, only four new classes of antibiotics have been brought to market in the past four decades (Cooper and Shlaes 2011). This falls far short of demand and only 74 years after the first treatment with 'natural' antibiotics, we again face the specter of incurable bacterial infections, now due to multidrug resistant pathogens (Cooper and Shlaes 2011). To prevent the post-antibiotic era from coming true, the discovery of novel antibiotic lead structures is key (Fowler *et al.* 2014). The chances to discover such structures correlate among other factors directly with the phylogenetic distance between the microorganism under study and the already known producers (Müller and Wink 2014). Thus, phylogenetically distinct bacterial linages might represent a valuable source for novel secondary metabolites. For instance, the candidate phylum 'Tectomicrobia' that dwells in association with a marine sponge was recently found to comprise a distinct and promising metabolic repertoire (Wilson *et al.* 2014). Besides such phylogenetic aspects, most potent antibiotic producers are characterized by large genomes with often more than 8 MB and complex life styles, involving for example differentiation processes (Müller and Wink 2014). Employing these criteria, entire bacteria phyla could be judged as putative 'talented' producers if basic knowledge about their ecology, cell biology and genomic architecture is available.

Thus, we screened the literature with a focus on little studied 'conspicuous' bacterial phyla that might represent putative 'talented' producers and identified Planctomycetes as candidates. Firstly, these ubiquitous and environmentally important bacteria possess large genomes (Glöckner *et al.* 2003, Jeske *et al.* 2013). Secondly, the planctomycetal cell biology is remarkable and was speculated to parallel eukaryotic cells in some aspects (Fuerst and Sagulenko 2011). While some of these findings, such as the lack of peptidoglycan cell walls have been recently challenged (Jeske *et al.* 2015, van Teeseling *et al.* 2015), other traits like their lifestyle switch after cell division, makes them unique among bacteria. Planctomycetes divide mostly through polar budding rather than binary fission, without employing the otherwise universal bacterial division protein FtsZ (Jogler *et al.* 2012, Lee *et al.* 2009, Pilhofer *et al.* 2008, Tekniepe *et al.* 1981). In the model organism *Planctopirus limnophila* for example, cell division is further coupled to a lifestyle switch, since only sessile stalked mother

cells can divide. Flagellated, planktonic daughter cells need to develop into stalked, sessile cells prior to division (Gade *et al.* 2005, Jogler *et al.* 2011, Jogler and Jogler 2013). Attached Planctomycetes can form dense biofilms, preferably on the surface of blooming diatoms (Morris *et al.* 2006, Pizzetti *et al.* 2011b) and they represent up to 50% of the bacterial community on *Laminaria hyperborea* (Bengtsson and Øvreås 2010). Given the slow growth of Planctomycetes under favorable laboratory conditions, some species have doubling times of up to one month (Strous *et al.* 1998), their abundance in such embattled carbon rich habitats in nature, in contrast to the largely oligotrophic surrounding water, appears counter intuitive (Lage and Bondoso 2014). Most other heterotrophs that dwell in such ecological niches divide much faster (for example 1.2 - 6.3 h for *Roseobacter* spec. (Christie-Oleza *et al.* 2012)) and should outcompete Planctomycetes. However, the phototroph–planctomycetal allelopathic interactions might involve the production of various secondary metabolites that might exhibit antimicrobial activity (defense against other, faster growing, heterotrophic bacteria) or algicidal (to destroy algae, diatoms or cyanobacteria for scavenging). The latter is supported by a positive correlation of planctomycetal abundance with chlorophyll a concentrations, pointing towards remineralization of algal biomass after blooming events through Planctomycetes (Pizzetti *et al.* 2011a).

Planctomycetes were found to possess secondary metabolite gene clusters employing genome mining, targeting nonribosomal peptide synthetases (NRPS) and polyketide synthases (PKS) encoding genes (Donadio *et al.* 2007). In the same year, planctomycetal type I polyketide keto-synthase domains were identified in two costal Antarctic sediments (Zhao *et al.* 2008). Only recently, we followed a holistic approach and combined comprehensive genome mining with physiological studies to improve our understanding of the planctomycetal potential to degrade algal polysaccharides and to produce secondary metabolites (Jeske *et al.* 2013). We further supported the hypothesis of allelopathic interactions between Planctomycetes and phototrophs and found, from a genomic perspective, Planctomycetes to be 'talented' producers.

In this study we employed chemical assays with planctomycetal crude extracts and biological screening methods for live visualization to prove the planctomycetal production of molecules with antibiotic activity. We further developed the entire set of tools ranging from cultivating marine and limnic Planctomycetes in chemical defined media within process-controlled bioreactors, to the preparation of planctomycetal extracts, their chromatographic separation, fractionation and biological screening. Here we provide the starting point for the in depth investigation of novel planctomycetal antibiotics.

MATERIAL & METHODS

General Cultivation of Microorganisms

In this study, the novel chemically defined Maintain Medium 1 (MM1) was developed for *Planctopirus limnophila* DSM 3776. MM1 consists of artificial freshwater (10 µM NH_4Cl, 10 µM KH_2PO_4, 100 µM KNO_3, 200 µM $MgSO_4 \cdot 7H_2O$, 100 µM $CaCl_2 \cdot 2H_2O$, 250 µM $CaCO_3$ and 300 µM $NaHCO_3$), that was supplemented with 20 mL/L mineral salts solution and 5 mL/L vitamin solution (double concentrated) and buffered with either 10 mM HEPES or 100 mM sodium phosphate buffer at pH 7.2. While HEPES was added before autoclaving, the sodium phosphate buffer ingredients (200 mM NaH_2PO_4 H_2O; 200 mM $Na_2HPO_4 \cdot 7H_2O$) were separately autoclaved and added afterwards (28 mL/L of mono salt solution and 72 mL/L di-salt solution). Mineral salt solution and vitamin solution were prepared according to DSMZ medium 621 (www.dsmz.de). The metal salts for the mineral salt solution consisted of 250 mg/L Na-EDTA, 1095 mg/L $ZnSO_4 \cdot 7$ H_2O, 500 mg/L $FeSO_4 \cdot 7$ H_2O, 154 mg/L $MnSO_4 \cdot H_2O$, 39.5 mg/L $CuSO_4 \cdot 7$ H_2O, 20.3 mg/L $CoCl_2 \cdot 6$ H_2O, 17.7 mg/L $Na_2B_4O_7$ $\cdot 10$ H_2O of which 50 mL were added per liter of mineral salt solution. To allow growth in MM1, 10 mL/L sterile filtered carbon source (2.5% glucose solution or 5% *N*-acetyl-D-glucosamine solution) was added.

For *Rhodopirellula baltica* SH1 DSM 10527*,* the chemically defined Maintain Medium 2 (MM2) was developed in this study. MM2 is composed of 250 mL/L 2x artificial sea water (46.94 g/L NaCl, 7.84 g/L Na_2SO_4, 21.28 g/L $MgCl_2 \cdot 6H_2O$, 2.86 g/L $CaCl_2 \cdot 2H_2O$, 0.384 g/L $NaHCO_3$, 1.384 g/L KCL, 0.192 g/L KBr, 0.052 g/L H_3BO_3, 0.08 g/L $SrCl_2 \cdot 6H_2O$ and 0.006 g/L NaF) supplemented with 5 mL/L 1M Tris/HCl pH 7.5, 20 mL/L and 5 mL/L vitamin solution (double concentrated, added after autoclaving) while buffered at pH 7.5. To allow growth in MM2, 40 mL/L sterile filtered carbon source (2.5% glucose solution or 5% *N*-acetyl-D-glucosamine solution) was added.

For strain Pan216 the novel M1H NAG ASW culture broth was developed in this study. It is composed of 250 mL/L 2x artificial sea water (46.94 g/L NaCl, 7.84 g/L Na_2SO_4, 21.28 g/L $MgCl_2 \cdot 6H_2O$, 2.86 g/L $CaCl_2 \cdot 2H_2O$, 0.384 g/L $NaHCO_3$, 1.384 g/L KCl, 0.192 g/L KBr, 0.052 g/L H_3BO_3, 0.08 g/L $SrCl_2 \cdot 6H_2O$ and 0.006 g/L NaF), supplemented with 0.25 g/L peptone, 0.25 g/L yeast extract, 20 mL/L mineral salts solution, 20 mL/L 5% (w/v) *N*-acetyl-D-glucosamine solution, 10 mL/L 2.5% (w/v) glucose and 5 mL/L vitamin solution (double concentrated), 1 mL/L trace element solution (1500 mg/L $N(CH_2COONa)_3$ x H_2O, 500 mg/L $MnSO_4$ x H_2O, 100 mg/L $FeSO_4 \cdot 7H_2O$, 100 mg/L $Co(NO_3)_2 \cdot 6H_2O$, 100 mg/L $ZnCl_2$, 50 mg/L $NiCl_2 \cdot 6H_2O$, 50 mg/L H_2SeO_3, 10 mg/L $CuSO_4 \cdot 5H_2O$, 10 mg/L $AlK(SO_4)_2$ x $12H_2O$, 10 mg/L H_3Bo_3, 10 mg/L $NaMoO_4 \cdot 2H_2O$, 10 mg/L $Na_2WO_4 \cdot 2H_2O$) and buffered with 10 mM HEPES at pH 8.0.

All strains were cultivated at 28°C in their respective culture broth with slight agitation (80 rpm).

Indicator strains were cultured according to DSMZ cultivation

recommendations(https://www.dsmz.de/catalogues/dzif-sammLung-der dsmz/indikatorstaemme-fuer-wirkstoffforschung.htmL.)

Phylogeny of Strain Pan216

Pan216 was identified by 16S rRNA gene sequencing after amplification with the modified universal primers 8f (5'–AGA GTT TGA TCM TGG CTC AG–3') and 1492r (5'–GGY TAC CTT GTT ACG ACT T–3') (Lane 1991). PCR reactions were performed directly on single colonies or liquid cultures using the *Taq* DNA Polymerase (Qiagen) with one reaction of 25 µL containing 11µL PCR–grade H_2O, 2.5 µL 10x CoralLoad buffer, 2.5 µL Q-Solution, 0.5 µL dNTPs (10 mM each), 1 µL sterile bovine serum albumin solution (20 mg/mL), 0.5 µL $MgCl_2$ solution (25 mM), 0.125 µL *Taq*–Polymerase (1U/µL) and 1 µL of each primer (10 pmol). The employed protocol consisted of two steps, the first step with an initial denaturation at 94°C, 5 min, 10 cycles of denaturation at 94°C, 30 sec, annealing at 59°C, 30 sec, elongation at 72°C, 1 min, followed by the second step with 20 cycles denaturation at 94°C, 30 sec, annealing at 54°C, 30 sec, elongation at 72°C, 1 min, and a final elongation step at 72°C, 7 min. All PCRs were carried out in an Applied Biosystems® Veriti® thermal cycler (Thermo Fisher Scientific) and PCR products were stored at 4°C until Sanger-sequencing.
Near full length 16S sequences were generated by assembly of the resulting sequences with the ContigExpress application of the Vector NTI® Advance 10 software (Thermo Fisher Scientific).
Alignments of near full length 16S rRNA sequences were performed using the SINA web aligner (Pruesse *et al.* 2012), corrected manually and used for phylogenetic tree reconstruction (Figure S1). Tree reconstruction was performed with the ARB software package (Ludwig *et al.* 2004) using the Maximum likelihood RAxML module and rate distribution model GTR GAMMA running the rapid bootstrap analysis algorithm, the Neighbor Joining tool with Felsenstein correction for DNA and Maximum Parsimony method employing the Phylip DNAPARS module. Bootstrap values for all three methods were computed with 1,000 resamplings including the *E. coli* 16S rRNA gene positions 101-1438. The analysis involved a total of 37 nucleotide sequences of described type strains and outgroup strains (grey box) (compare Table S2).

Growth Measurement of Planctomycetes

To measure growth of *P. limnophila* in the different cultivation media (Figure 1 A), 250 mL baffled flasks with 70 mL respective growth medium were inoculated with 10 mL pre-culture, which were washed twice with MM1 prior inoculation. Likewise, *R. baltica* and Pan216 were incubated with different carbon sources (Figure 1 B and 1 C) and inoculated with a pre-culture washed twice with MM2. Afterwards, the respective carbon source was supplemented (4 mL of 2.5% glucose, 4 mL of 2.5% dextran or 2 mL of 5% *N*-acetyl-D-glucosamine). As control *P. limnophila* was grown in M1 and *R. baltica* in M2 medium (Jeske *et al* 2013) while Pan216 was grown in M1H NAG ASW medium. Individual

growth conditions were analyzed in three biological replicates. The optical density of the culture was measured every eight and 16 hours at 600 nm with a spectrophotometer.

Cultivation of Planctomycetes in a Bioreactor

The respective growth medium was autoclaved in a 368 L chamber autoclave (BeliMed) within the 10 L culture vessel of the bioreactor (UniVessel, Sartorius). After sterile supplementation of the respective vitamins and carbon source the reactor was incubated for two days to exclude contaminations. For sterile inoculation, a planctomycetal pre-culture (0.1 volume) was transferred via a culture bottle into the 10 L culture vessel of the BIOSTAT®B system.

P. limnophila was cultured at 28°C in MM1 supplemented with glucose with a constant pH of 7.2 at 100 rpm agitation and a constant air supply (0.5 bar, 0.85 L/min). Samples for glucose consumption, OD_{600} and biomass determination were taken every 24 hours (Figure 3 A).

In contrast, *R. baltica* was cultured in MM2 supplemented with glucose at pH 7.5 with otherwise identical parameters (Figure 3 B).

For fed-batch cultivation the *P. limnophila* culture was spiked after 7 days with 25 mL *N*-acetyl-D-glucosamine (NAG) and 10 mL vitamin solution and cultured for ten more days (Figure S3).

Extraction of Secondary Metabolites from Planctomycetes

For crude extract preparation, 600 mL of a *P. limnophila* culture were incubated with 2% (v/v) of purified adsorbent resin XAD-16N (Rohm and Haas) at 28°C with 80 rpm agitation in a baffled flask for 3 days. The XAD-16N was collected by filtration through an analysis sieve and transferred into an extraction flask. 200 mL acetone were added and incubated in darkness for one hour at room temperature. To remove the XAD resin the eluate was filtered (Macherey & Nagel 615 ¼, pore size 4-12 µm) after incubation. In parallel, cells were harvested by centrifugation and suspended in 200 mL acetone for one hour in darkness at room temperature, followed by filtration. Employing a rotary evaporator (Hei VAP-Precision Heidolph) the acetone was removed to yield a solid residue (36°C; at 130 rpm; 1 mbar). The obtained residues were dissolved in 1 mL methanol (pA) and stored at -20 °C. Extracts for *R. baltica* and Pan216 were prepared in the same manner. Cultures of Pan216 and XAD were harvested after 5 days of incubation due to their slower growth.

Measurement of Biomass Production and Glucose Consumption

To determine the dry weight of the planctomycetal cells at the given time points, 25 mL of the respective culture were filtered through two superimposed filters with a pore size of 2.5 µm each (Whatman Grade 5 Qualitative Filter Paper) employing a vacuum pump operating at 800 mbar. The filters were subsequently dried for 60 min at 80°C. Comparison of filter weight before and after filtration gave the bacterial dry mass. The glucose concentration was determined with a test strip (MQuantTM) according to the manufacturer's description.

Minimum Inhibitory Concentration Assay

To examine the minimum inhibitory concentrations (MIC) of planctomycetal crude extracts, 26-56 µL of indicator bacteria (for detailed information see Table S1) were added to 20 mL of their respective culture medium and mixed well. The indicator strain stocks contained each 1 mL bacteria suspension with an OD_{600} of 3.54 for *Escherichia coli* DSM 1116, 4.05 for *Escherichia coli* TolC, 7.7 *Micrococcus luteus* DSM 1790, 3.69 for *Bacillus subtilis* DSM 10 and 3.86 for *Staphylococcus aureus* DSM 346. Subsequent aliquots were stored at -80°C until usage. 150 µL of the diluted culture was dispensed into each well of a 96-well microtiter plate (initial OD_{600} for the indicator bacteria was 0.01). An additional 130 µL of diluted indicator culture was added to the first row. 20 µL of the planctomycetal crude extracts were added to the first row. A serial dilution of the extract (1:1) was made by transferring 150 µL from one well to the next, this was done from row A to H. For negative and positive controls either 20 µL methanol or 20 µL of the respective culture medium were added instead of planctomycetal crude extracts. Subsequently, the microtiter plates were incubated for 24 hours under constant agitation (150 rpm) at 30°C or 37°C. After incubation, the growth of the indicator strains was reviewed visually. No turbidity of the media in a well showed an inhibition of the indicator strain.

Agar Plate Diffusion Assay

To determine antibiotic activity, filter discs (pore size 4-7 µm; Whatman No 597) were coated with 90 µL of planctomycetal crude extracts, while methanol coated discs served as negative control. The treated discs were placed on soft-agar plates inoculated with *B. subtilis* and incubated over night at 28°C. Soft-agar plates consisted of a 10 mL 1.5% LB agar layer covered with a second layer of 10 mL 0.7% LB agar inoculated with 100 µL of a *B. subtilis* overnight culture.

Dual Culture Assay

Dual culture assays were performed on M2 1.5% agar plates with *R. baltica* producer and *E. coli* DSM 1116 as indicator strain. Given the different doubling times of both species, *R. baltica* was inoculated first and incubated for two days at 28°C. Afterwards, *E. coli* was inoculated on the same plate and the agar dishes were incubated for three more days at 28°C. To investigate if growth-altering effects appear in a distance-dependent manner, respective bacteria were plated at different defined distances from each other (2 mm, 10 mm, and 15 mm respectively).

HPLC-Analysis of Planctomycetal Extracts

High-performance liquid chromatography (HPLC) analysis was performed with a Prominence HPLC (Shimadzu) equipped with a Macherey & Nagel, CC 250/4.6 NUCLEODUR 100-5 C18 column (pore size 100 Å, particle size 5 µm) coupled to a SPD-M20A photo diode array (PDA) detector (Shimadzu). The PDA detector recorded all emitted wavelengths, while the wavelength with the highest signal intensities (254 nm) was used for further discrimination of the extracts. 15 µL of the investigated extracts were injected and eluted with a modified methanol-water gradient (solvent A: H_2O; solvent B: methanol, gradient: 5% B for 10 min increasing to 100% B in 15 min, maintaining 100% B for 30 min, flow rate 0.6 mL/min). Prior to the analysis of the extracts, the column was equilibrated with pure methanol. Results were analyzed using the LCsolution Shimadzu Software.

HPLC Fractionation

An Agilent 1260 Series HPLC-UV system equipped with a Waters, XBridge BEH C18, 2.1x100 mm column (pore size 135 Å, particle size 3.5 µm) was used for the chromatographic fractionation of crude extracts. The same HPLC gradient was used as for the high-resolution electrospray ionization mass spectrometry (HRESIMS) instrument (described below). The flow through was collected in 30 sec intervals into a 96-well microtiter plates. Afterwards, the plates were dried by a constant nitrogen-flush for 40 min, inoculated with 150 µL indicator bacteria per well and incubated as described above. After 24 hours the plates were evaluated and documented employing a custom-made mirror stand and a CANON EOS 10D digital camera.

Mass Spectrometry

Electrospray ionization mass spectrometry (ESIMS) spectra in positive and negative mode were obtained with an Agilent 1260 Infinity Series HPLC-UV system (column 2.1 × 50 mm, 1.7 µm, C18 Acquity UPLC BEH (Waters), solvent A: H2O + 0.1% formic acid; solvent B: ACN + 0.1% formic acid, gradient: 5% B for 0.5 min increasing to 100% B in 19.5 min, maintaining 100% B for 5 min, flow rate 0.6 mL/min, UV detection 200–600 nm) equipped with a diode array detector and an ESIiontrap MS detector (Amazon, Bruker).

HRESIMS spectra were obtained with an Agilent 1200 series HPLC-UV system (solvent A: H_2O/ACN (95/5) + 5 mM/L of NH_4Ac + 40 µL/L of acetic acid; solvent B: H_2O/ACN (5/95) + 5 mM/L of NH_4Ac + 40 µl/l of acetic acid, gradient: 10% B increasing to 100% B in 30 min, maintained 100% B for 10 min; flow rate (FR)=0.3 mL/min; UV detection 200 - 600 nm) combined with an ESI-TOF-MS (Maxis, Bruker; scan range 100-2500 m/z, rate 2 Hz, capillary voltage 4500 V, dry temperature 200°C).

RESULTS

Chemically Defined Growth Conditions for Marine and Limnic Planctomycetes

We selected the marine species *Rhodopirellula baltica* SH1 DSM 10527 and the limnic organism *Planctopirus limnophila* DSM 3776 as model Planctomycetes together with the phylogenetically-distant and recently isolated strain Pan216 (Figure S1) for subsequent experiments. For both established model species, optimized growth conditions in complex media, containing rather high amounts of yeast extract and peptone, have been previously described (Jeske *et al.* 2013, Jogler *et al.* 2011). While the optimal growth conditions in rich media for strain Pan216 were determined in this study (see material and methods).

To enable ecomimetic experiments that mimic certain natural conditions in the laboratory, and to optimize HPLC detection and fractionation, we developed a chemically-defined and carbon source free maintain medium for limnic- (MM1) and marine conditions (MM2). *P. limnophila*, *R. baltica*, and Pan216 cells survived under such conditions, but were not able to divide and grow without supplementation of any maintain medium with a carbon source (Figure 1, red curves). After a starvation period (5 to 9 days) in the respective Maintain Media, all three strains were able to fully recover when specific carbon and nitrogen sources were added. Remarkably, *P. limnophila* grew even more efficiently in MM1 + glucose (same growth rate but higher biomass attained) (Figure 1 A, blue curve) or MM1 + *N*-acetyl-D-glucosamine (lower growth rate but higher biomass attained) (Figure 1 A, pink curve), than in the originally described isolation medium (Figure 1 A green curve). *R. baltica* grew less efficiently with MM2 + glucose (Figure 1 B, blue curve) or MM2 + *N*-acetyl-D-glucosamine (Figure 1 B, pink curve) and did not reach the same OD_{600} compared to the medium culture broth M2 (Figure 1 B green curve). Pan216 grew best if fed with M1H NAG ASW medium developed in this study (Fig 1C green curve). The chemically-defined MM media were also supplemented with other carbon sources such as the complex polysaccharide dextran (Figure 1, orange curves) that provided growth for *P. limnophila* and Pan216 but not for *R. baltica.* In the case of *R. baltica*, the supplementation of *N*-acetyl-D-glucosamine (Figure 1 B, pink curve) led to initial biofilm formation resulting in an early decrease of the cultures optical density. This effect was even stronger after adding dextran (Figure 1 B, orange curve) and explains why the optical density was lower than in the control culture (Figure 1 B, red curve) without any carbon source added. Thus certain carbon sources might trigger a lifestyle switch from planktonic growth to biofilm formation.

The adsorber resin XAD binds unspecifically to both secreted secondary metabolites and components of the complex growth media M1 and M2, resulting in visible media component signals in HPLC measurements even when no-bacteria medium control samples were measured (Figure S2 A and C). However, extraction of our newly developed MM1 and MM2 media with XAD showed no media component signals in HPLC analysis (Figure S2 B and C), making them the preferred choice to investigate the influence of trigger substances on secondary metabolite production in Planctomycetes.

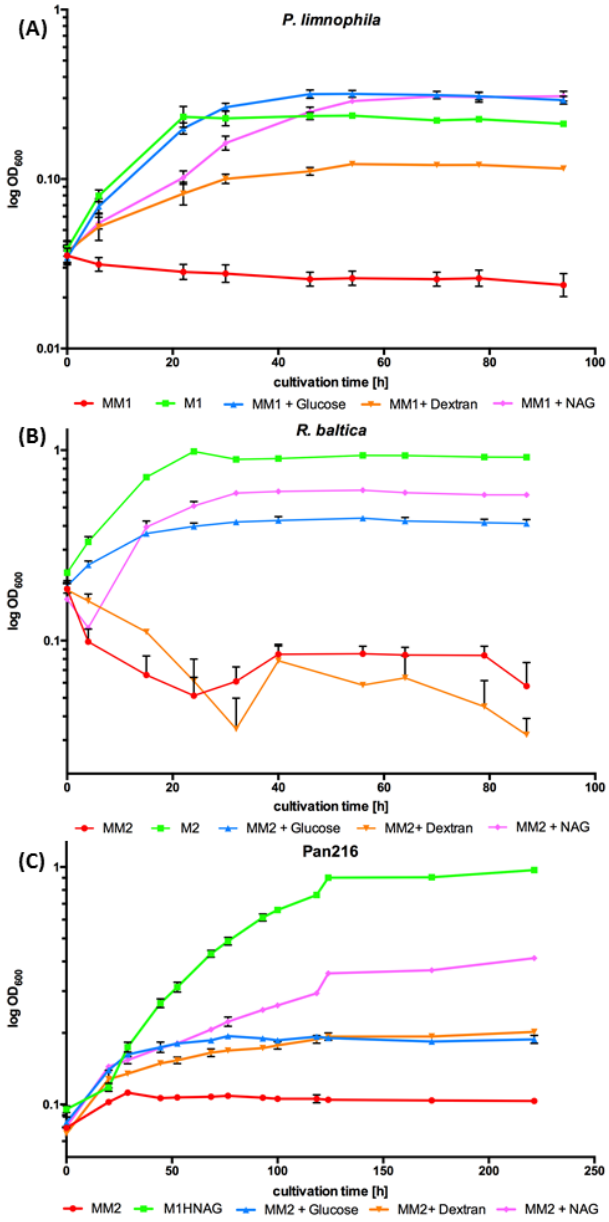

Figure 1: Growth of *P. limnophila, R. baltica* and isolate Pan216 under different cultivation conditions
Logarithmic OD_{600} values as measure of growth over 96 hours for **A**: *P. limnophila*,
B: *R. baltica* and 225 hours for **C:** Isolate Pan216 under different cultivation condition: M1 and M2: complex culture media containing yeast extract, peptone and glucose (green curves). Maintain medium 1 or Maintain medium 2 (MM1 and MM2) are chemically defined but lack a carbon source and thus, unsupplemented, do not allow for bacterial growth of Planctomycetes (red curves). MM1 and MM2 were supplemented with glucose (blue curves), dextran (orange curves) or N-acetyl-D--glucosamine (NAG) (pink curves) to allow growth of *P. limnophila*, *R. baltica* and isolate Pan216. Each time point represents the mean of triplicate measurements.

Ecomimetic Cultivation of Planctomycetes

The chemically-defined Maintain Media (MM) were developed to enable ecomimetic experiments which attempt to recreate environmental situations under defined conditions in the laboratory. The rational was that certain environmental cues, such as a particular carbon source, would stimulate secondary metabolite production in Planctomycetes, and consequently alter the peak profile in subsequent HPLC analysis. For a proof of principle, we simulated the planctomycetal interaction with cyanobacteria by adding a key component of bacterial cell walls -*N*-acetyl-D-glucosamine (NAG)- to our MM. Since cyanobacteria occur in both, marine- and limnic habitats, we performed the evaluation of our MMs with *R. baltica* (marine) and *P. limnophila* (limnic) in MM2 and MM1 respectively. After cultivation with XAD for three days at 28°C, XAD extracts were analyzed *via* HPLC. For *P. limnophila*, three minor peaks were exclusively detected when cells were cultivated with glucose as sole carbon source (Figure 2 A, black asterisk), while one peak was unique to cultivation with NAG as sole carbon source (Figure 2 A, red asterisk). When cultivated with glucose, *R. baltica* produced secondary metabolites that led to five major and five minor peaks in the HPLC chromatogram (Figure 2 B, green curve). In contrast, when fed with NAG, the subsequent HPLC chromatogram contains two additional minor peaks, (Figure 2 B, black arrows) while one minor peak vanished (Figure 2 B, black asterisk). Three of the major peaks were significantly higher (Figure 2 B, red asterisk), while one showed less signal intensity (Figure 2 B, red arrow). The overall signal intensity of *P. limnophila* extracts showed less variation between the cultivation conditions than *R. baltica* did. However, comparison of extracts from *P. limnophila* (Figure 2 C and D, blue) and *R. baltica* (Figure 2 C and D, red) showed that both organisms display entirely different HPLC peak patterns.

Thus our Maintain Media allow ecomimetic experiments that lead to the production of different metabolites in response to different carbon sources.

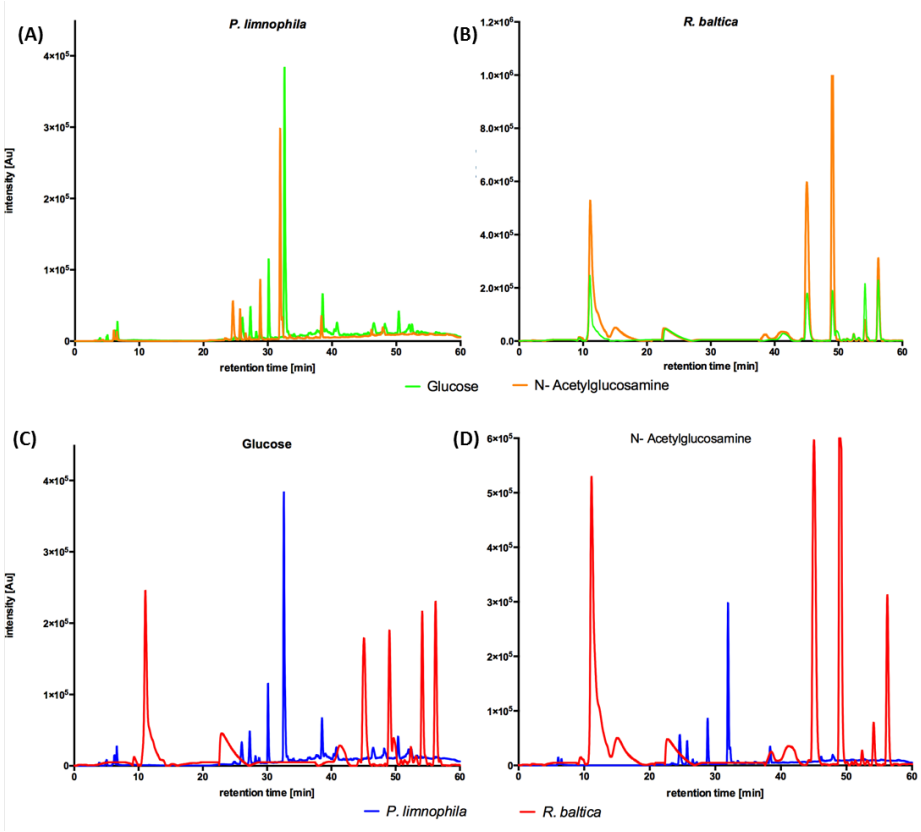

Figure 2: HPLC- analysis of *R. baltica* and *P. limnophila* extracts

A: HPLC-analysis of extracts generated from *P. limnophila* cultivated in MM1 supplemented with glucose (green curve) and *N*-acetyl-D-glucosamine (NAG; orange curve). Feeding glucose resulted in higher major peaks than NAG. Three minor peaks were exclusively produced if glucose was added (black asterisk), while one major peak was unique to NAG cultivation (red asterisk) **B:** HPLC-analysis of extracts generated from *R. baltica* cultivated in MM2 supplemented with glucose (green curve) or NAG (orange curve). The intensity of the secondary metabolite peaks was higher in extracts from *R. baltica* cultivated with NAG as carbon source compared to extracts from cultivation with glucose. Extracts from glucose cultivation showed five peaks after twelve minutes and 40 to 56 minutes with intensity only up to 2.2 $x10^5$ Au, while same signal peaks from extracts from NAG cultivation showed intensity up to 10^6 Au. Three of the major peaks were significantly higher (red asterisk), while one showed less signal intensity (red arrow). The HPLC spectrum contains two additional minor peaks (black arrows) while one minor peak vanished (black asterisk). **C:** Comparison of HPLC-chromatograms of XAD extracts from *P. limnophila* cultivated in MM1 (blue curve) and *R. baltica* in MM2 (red curve) supplemented with glucose as sole carbon source. Secondary metabolite signals from *P. limnophila* extracts were detected after a retention time of seven minutes and 26 to 38 minutes with an intensity of 5 $x10^4$ up to 3.8 $x10^5$ Au, while for *R. baltica* a signal was detected after ten minutes with an intensity of 2.5 $x10^5$ Au and additional signals were detected after 44 to 57 minutes with an intensity of 1.8 $x10^5$ up to 2.4 $x10^5$ Au. **D:** Comparison of HPLC- chromatograms of XAD extracts from *P. limnophila* cultivated in MM1 (blue curve) and *R. baltica* in MM2 (red curve) supplemented with NAG as sole carbon source. Secondary metabolite signals from *P. limnophila* were detected with intensity up to 3 $x10^5$ Au after a retention time of 25 to 38 minutes while secondary metabolite peaks from *R. baltica* extracts after twelve and 40 to 56 minutes with intensity up to 10^6 Au.

Cultivation of Planctomycetes in Process Controlled Bioreactors

Thus far we have presented tools and procedures for the discovery of planctomycetal bioactive molecules, yet one important feature is missing if they are to be useful: large scale cultivation. Anammox Planctomycetes are routinely cultivated in different types of bioreactors in small, large and industry scale, mainly for waste water treatment (for review see (van Niftrik and Jetten 2012)). However, little to no experience exists with the planctomycetal orders Phycisphaerales and Planctomycetales. The known species belonging to the order Phycisphaerales have small genomes with few secondary metabolite related clusters while species of the order Planctomycetales were found to comprise large genomes with many secondary metabolite related genes (Jeske *et al.* 2013). Among Planctomycetales, only the cultivation of *R. baltica* in a small-scale custom-made chemostat was described thus far (Frank *et al.* 2011). However, such experiments were not performed with the intention to obtain secondary metabolites.

In this study, we developed the required media and procedures to cultivate both limnic (*P. limnophila*) and marine (*R. baltica*) Planctomycetes in a 10-liter bench-top bioreactor (BIOSTAT®B, Sartorius). Our protocols (see material and method section for details) fit to batch (Figure 3) and fed-batch (Fig S3) cultivation in different sized stirring tank reactors as our reactor system allows seamless scale-up, at least for up to 100 liters.

First, we determined optimal growth conditions in maintain medium supplemented with glucose (Figure 3 A and B) and obtained optimal biomass production, whereby the pH was kept constant at 7.5 for *R. baltica* and at 7.2 for *P. limnophila*. Since HEPES is used routinely to buffer planctomycetal culture media, the future scale-up might be limited by high costs of this chemical. Thus, we explored different buffer systems and found that at least the growth of *P. limnophila* is not affected by phosphate buffering (material and methods). Growth was measured by OD_{600} and dry weight, while glucose consumption was determined in addition (Figure 3 A+B). While both curves, dry weight and OD_{600}, comprise a similar shape for *R. baltica*, the slope of the OD_{600} curve for *P. limnophila* is steeper than the curve representing dry weight measurements.

Paralleling the extraction procedure employed for flask cultures, we added XAD to the bioreactor that was finally harvested with an analyze sieve prior to extraction. Thus, even for planctomycetal large-scale cultures our procedure allows small volume downstream processes, significantly reducing the amount of organic solvents and effort for the extraction process.

When two independent *P. limnophila* 5 L cultivation batches were employed for XAD extraction and subsequent HPLC analysis, the resulting metabolite profiles differed slightly (Figure S4). Eight minor peaks (red asterisks) and one major peak (black asterisks) occurred only in one of the two replicates, while only the three major peaks (black arrows) were found in both extracts.

Thus, we developed a large scale cultivation approach for aerobic Planctomycetes that reduces cost and effort but still lacks 100% reproducibility.

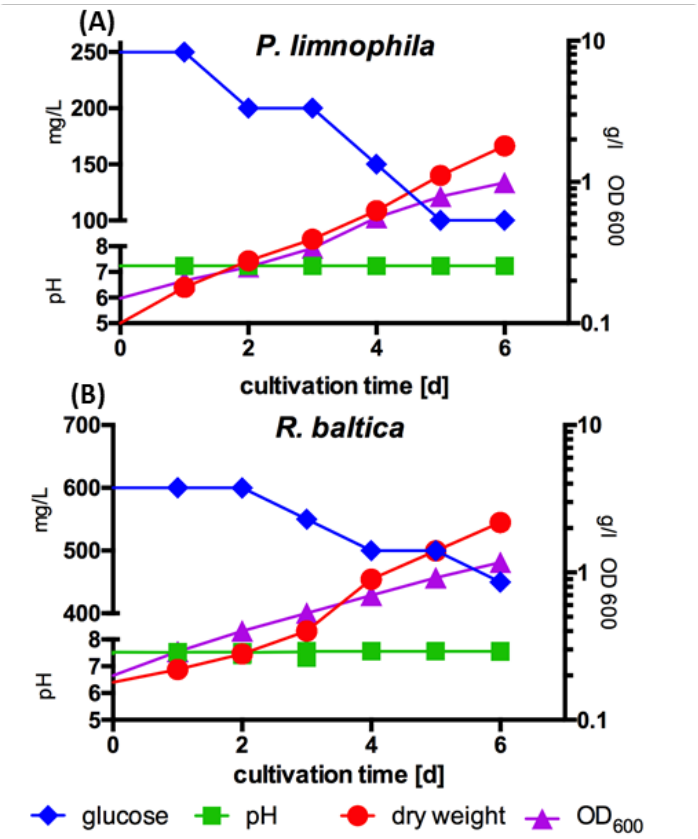

Figure 3: Measurement of biomass production compared to optical density and glucose consumption of *P. limnophila* and *R. baltica* at constant pH.
A: Biomass production of *P. limnophila* compared to optical density at 600 nm (OD_{600}) and glucose consumption at constant pH over six days in MM1 supplemented with glucose in a 10 L fermenter. Glucose concentration decreased from 250 mg/L to 100 mg/L (blue curve), while the OD_{600} increased from 0.15 up to 0.99 (purple curve) and correlating with the OD_{600} also the dry weight increased from 0.1 g/L up to 1.8 g/L (red curve) during cultivation. The pH was stabilized at 7.2 (green curve). **B**: Biomass production of *R. baltica* compared to, OD_{600}-value and glucose consumption at constant pH over six days in MM2 supplemented with glucose in a 10 L fermenter. Glucose concentration decreased from 600 mg/L to 450 mg/L (blue curve), while the OD_{600} increased from 0.2 to 1.170 (purple curve) and correlating with the OD_{600} also the dry weight increased from 0.18 g/L and to 2.18 g/L (red curve) during cultivation. The pH was stabilized at 7.5 (green curve).

Screening of planctomycetal extracts for antimicrobial activity

One aim of this study was to develop cultivation techniques that could stimulate the production of bioactive molecules with antimicrobial activity as demonstrated in the previous section. However, extracts obtained in such ecomimetic experiments require a screening procedure to determine potential antimicrobial activity. Thus, for the first time we demonstrate different screening methods for planctomycetal extracts and strains and

evaluated their usefulness.

The semi-quantitative agar plate diffusion assay was used to determine the inhibitory effect of *R. baltica* cell- (methanol) and XAD resin extracts obtained from *R. baltica* cultures against *B. subtilis*. While the negative control and the cell extract discs showed no influence on bacterial growth, the planctomycetal XAD extract displayed a zone of *B. subtilis* growth inhibition, pointing towards an antibiotic molecule produced by *R. baltica* (Figure 4 A).

The dual culture assay revealed that reducing the distance between *R. baltica* and *E. coli* K12 increases the growth inhibition effect (Figure 4 B). *E. coli* grew to highest density with the maximum chosen distance of 15 mm. When the distance to *R. baltica* was reduced to 10 mm, the *E. coli* grew less well. Ultimately, only minor growth of *E. coli* was observed when the distance was reduced to of 2 mm distance.

The minimum inhibitory concentration assay (MIC) was performed for *R. baltica* extracts (Figure 4 C). Several dilution series were prepared against *E. coli* TolC, *S. aureus* and *M. luteus*. Only the highest concentration of *R. baltica* XAD extract in the first well showed an effect against the *E. coli* TolC strain (Figure 4 C: XAD). However, a strong inhibitory effect against *S. aureus* was shown with the *R. baltica* XAD extract (Figure 4 C: XAD). The first three wells of the dilution series showed a distinct inhibition of growth. The lowest concentration in which the bacterial growth was inhibited therefore contained the antibiotic at minimum inhibitory concentration. In contrast, *S. aureus* treated with the cell extract grew normally and showed no signs of growth inhibition (Figure 4 C: cell).

Likewise, only the XAD extract had a strong inhibitory effect against *M. luteus* in the first four wells (Figure 4 C: XAD), whereas the cell extract had no effect on *M. luteus* (Figure 4 C: cell). However, since no bacterial growth could be detected in the first two wells of the dilution series for the negative control, the effect of the first two wells in the XAD column might be caused by methanol rather than by a potential antibiotic within the crude extract. However, bacteria were not inhibited in the third well of the negative control, so extracts with activity at this dilution could still contain an active compound.

Thus, all three evaluated screening methods, agar plate diffusion, dual culture, and MIC assay were useful to analyze planctomycetal antimicrobial activity. For the first time we demonstrated antibacterial activity of *R. baltica* against both, Gram-negative and Gram-positive bacteria.

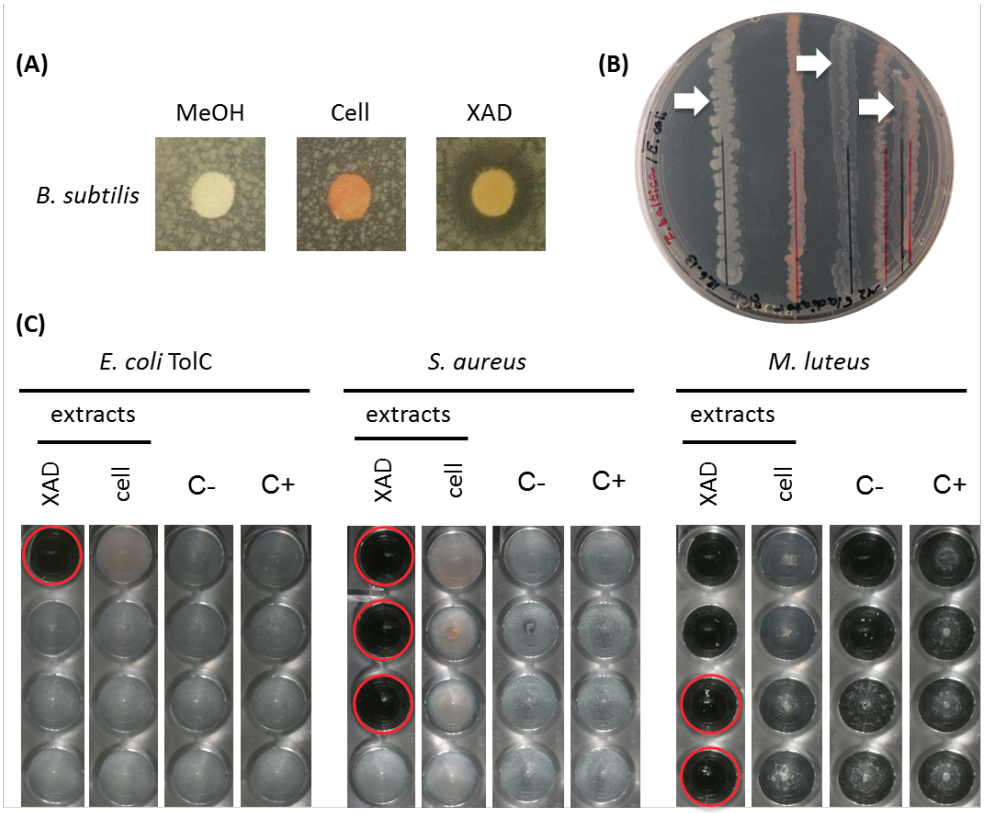

Figure 4: Activity assays of *R. baltica* methanol extracts.
A: Agar plate diffusion assay of *R. baltica* methanol cell extracts against *B. subtilis*. Discs are coated with 90 µL methanol (MeOH), *R. baltica* cell or XAD extract respectively and incubated over night on soft-agar plates inoculated with *B. subtilis*. The clear areas around the discs treated with XAD extract show a distinct zone of *B. subtilis* growth inhibition, while methanol or cell extract show no effect. **B:** Dual culture assay of *R. baltica* and *E. coli*. *E. coli* grew best with the maximal chosen distance of 15 mm to *R. baltica*. *E. coli* cells grew weaker when the distance was reduced to 10 mm and weakest at 2 mm (white arrows). **C:** Dilution minimum inhibitory concentration (MIC) assay with *R. baltica* cell and XAD extracts. Growth inhibitory effect of cell and XAD methanol extracts on: treated *E. coli* TolC deletion mutant, *S. aureus* DSM 346 and *M. luteus* DSM 1790 cells. **XAD**: Serial dilution column with XAD extract; **Cell**: Serial dilution column with cell extract; **C-**: Serial dilution column with methanol as negative control; **C+**: Respectively untreated cells as positive control. Red circles highlight inhibited cells. If the same methanol concentration also inhibited the growth, the effect of the extracts was not taken into account (see **C** first two wells of XAD extract). The last well in which no bacteria grow contains the antibiotic at its minimal inhibitory concentration.

Correlation of Antimicrobial Activity with Distinct HPLC Peaks

After development of ecomimetic cultivation, biological screening methods, and protocols to facilitate the analysis of planctomycetal antibiotic production, next the HPLC signals need to be correlated with biological activity to facilitate structure elucidation of the antibiotic molecules. Thus, we developed a procedure that allows correlation of HPLC peaks with antimicrobial activity of planctomycetal extracts. Again, a limnic (*P. limnophila*) and a marine species (strain Pan216) were chosen for the proof of principle study. After cultivation and extraction of culture with adsorbent resin, the MIC assay screening against indicator strains (Table S1) was performed. Once the analyzed extract showed an activity against one of the indicator strains (Figure 4 C and Figure 5), a semi preparative HPLC run was performed (Figure 5). Our integrated approach consists of utilizing HPLC instruments coupled with both HRESIMS as well as a fractionation collector for 96-well microtiter plates, which are used in a subsequent bioassay against the prior determined strains. Both HPLC systems were equipped equally, so that chromatograms and retention times can directly be compared. Thereby it is possible to assign the activity observed for the crude extract to individual peaks, and simultaneously to determine their molecular formula (Figure 5, Table 1). Furthermore, tandem mass spectrometry (MS/MS) and UV/Vis spectra can sometime provide early information about the chemical structure of the desired substance. Although the HPLC gradient and solvents can be varied, the use of a water/acetonitrile (ACN) system provided best chromatographic separation and we suggest this as standard procedure for the analysis of planctomycetal extracts. Since the use of formic acid did not lead to the reproducible detection of bioactive wells, we concluded that some of the bioactive compounds might be degraded under acidic conditions and consequently we used an acetate buffered solvent system instead.

Two replicate cultivation and extraction experiments were performed for both *P. limnophila* and strain Pan216.

Fractionation of *P. limnophila* replicate #1 resulted in inhibition of various wells. The obtained results were not reproducible and no activity could be assigned to peaks in the UV chromatogram.

In contrast, repeated fractionation of 15 µL extract each for *P. limnophila* replicate #2 led to reproducible inhibition of *B. subtilis* growth in well 'B6', which had been collected from 23-23.5 min (Table 1). The chromatogram showed a major peak at this retention time (Figure 5 A). The same peak was observed at a retention time of 22.4 min in the HRESIMS system. Its molecular formula $C_{26}H_{51}NO_6$ was deduced from its $[M+H]^+$ peak at *m/z* 474.3880 as well as its $[M+Na]^+$, $[2M+H]^+$, and $[2M+Na]^+$ peaks at *m/z* 496.3608, 947.7514, and 969.7325, respectively (Table 1). The assignment of the molecular ion cluster was further confirmed by the observation of the $[M+CO_2H]^-$ peak at *m/z* 518.3 in the ESIMS spectrum measured in negative mode. A search with the molecular formula $C_{26}H_{51}NO_6$ in the Dictionary of Natural Products (DNP) on DVD resulted in no known metabolites (Table 1).

In the case of strain Pan216, both replicates resulted in different inhibition patterns as well. For replicate #1 inhibition of wells B7, C7 and G7 was observed that correspond to three major peaks of the slightly lipophilic region of the chromatogram (Figure 5 B). These three peaks were detected in HRESIMS spectrum at 25.1, 25.8 and 27.6 minutes and molecular formulae of $C_{18}H_{30}O_2$, $C_{22}H_{32}O_2$ and $C_{22}H_{34}O_2$ were assigned from their $[M+H]^+$ and $[M+Na]^+$ peak pairs (Table 1). These assignments were further confirmed by $[M+H]^+$ $[M-H]^-$ pairs in positive/negative mode ESIMS spectra. The search for these molecular formulae in the DNP resulted in 122, 38 and 61 hits (Table 1).

Neither of those peaks were observed in the chromatogram of Pan216 replicate #2 nor was activity observed in wells B7-G7. Instead, well C6 (23.5-24 min) was constantly inhibited. The corresponding peak shows characteristic UV absorption maxima at 244 and 294 nm, which might suggest an aromatic residue. The $[M+H]^+$ peak in the HRESIMS spectrum at *m/z* 294.0532 could indicate the molecular formula as $C_{12}H_{11}N_3O_4S$, which would explain the observed +2 satellite in the molecular ion cluster as the ^{34}S isotope. However, the other possibilities for the molecular formula $C_{13}H_{12}NO_5P$ and $C_{20}H_7NO_2$ cannot be excluded. In the ESIMS spectra in negative mode $[M-H]^-$ respectively $[2M-H]^-$ peaks at 291.1 and 585.0 confirm the assignment. There is no known natural product included in the DNP for any of the 3 molecular formulae.

Thus we demonstrated a procedure to correlate HPLC peaks of planctomycetal extracts with antimicrobial activity that allows subsequent purification of the compound of interest. Initial data pointed towards novel bioactive molecules with antimicrobial activity produced by Planctomycetes.

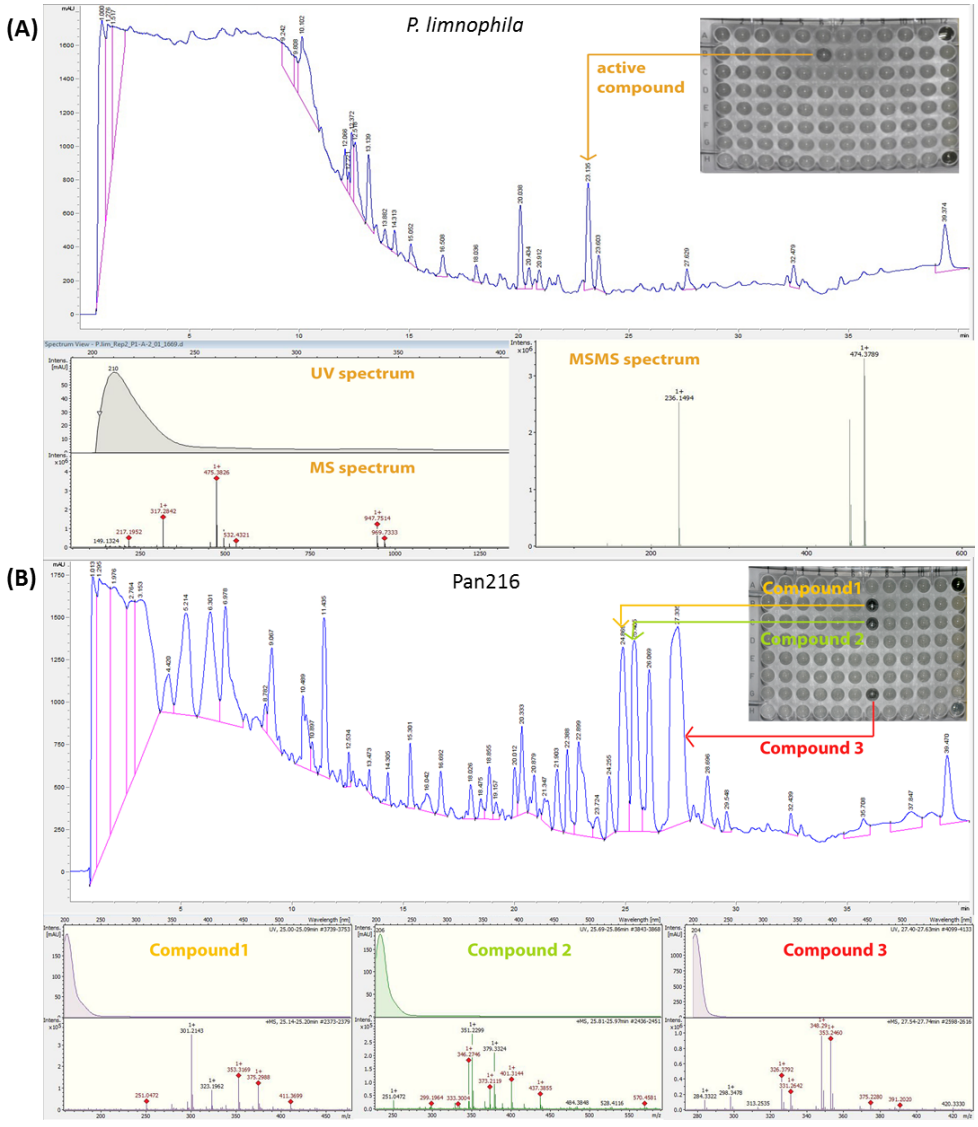
(A)
P. limnophila
active compound
UV spectrum
MS spectrum
MSMS spectrum
(B)
Pan216
Compound1
Compound 2
Compound 3

Figure 5: Fractionation analysis of planctomycetal crude extracts
A: Combined chromatogram of HPLC fractionations, UV/Vis, mass- and tandem mass (MS/MS) spectrometry to identify the active compound, which causes the growth inhibition of *P. limnophila* crude extract #2 against *B. subtilis* on a 96 well plate. Fractionation of 15 µL extract for *P. limnophila* replicate #2 led to reproducible inhibition of *B. subtilis* growth in well 'B6', which had been collected from 23-23.5 min. The chromatogram shows a major peak at this retention time. The same peak was observed at a retention time of 22.4 min in the HRESIMS system. Its molecular formula $C_{26}H_{51}NO_6$ was deduced from its $[M+H]^+$ peak at *m/z* 474.3880 as well as its $[M+Na]^+$, $[2M+H]^+$ and $[2M+Na]^+$ peaks at *m/z* 496.3608, 947.7514 and 969.7325, respectively (Table 1). The assignment of the molecular ion cluster was further confirmed by the observation of the $[M+CO_2H]^-$ peak at *m/z* 518.3 in the ESIMS spectrum measured in negative mode. **B:** Combined chromatogram of HPLC fractionations, UV/Vis, mass spectrometry to identify three active compounds, which causes growth inhibition of *B. subtilis* of Pan216 crude extract #1 in a 96 well plate. In case of strain Pan216 both replicates resulted in different inhibition patterns as well. Inhibition of wells B7, C7 and G7 was observed that correspond to three major peaks of the slightly lipophilic region of the chromatogram. These three peaks were observed in HRESIMS spectrum at 25.1, 25.8 and 27.6 minutes; molecular formulae of $C_{18}H_{30}O_2$, $C_{22}H_{32}O_2$ and $C_{22}H_{34}O_2$ were assigned from their $[M+H]^+$ and $[M+Na]^+$ peak pairs (Table 1). These assignments were further confirmed by $[M+H]^{+/}$ $[M-H]^-$ pairs in positive/negative mode ESIMS spectra. Wells A12 and H12 are negative controls treated with tetracycline and crude extract respectively.

Table 1: Antibacterial metabolites found in *P. limnophila* and Pan216

Organism	Rt [min]	*m/z*[a]	Molecular Formula	Hits in DNP	DBE	MS/MS	possible fragments
P.limnophila R2	22.4	**474.3880**	$C_{26}H_{57}NO_6$	0	2	456.3685	$C_{26}H_{50}NO_5$
		947.7514	$C_{52}H_{103}N_2O_{12}$			317.2845	$C_{22}H_{37}O$
		969.7333	$C_{52}H_{102}N_2O_{12}Na$			236.1495	$C_{10}H_{22}NO_5$
						217.1954	$C_{16}H_{25}$
Pan216 R1	27.6	**331.2642**	$C_{22}H_{35}O_2$	132	6	313.2532	$C_{20}H_{34}ONa$
		353.2461	$C_{22}H_{34}O_2Na$			295.2429	$C_{20}H_{32}Na$
						271.2429	$C_{18}H_{30}ONa$
						247.1703	$C_{14}H_{24}O_2Na$
						233.1551	$C_{13}H_{22}O_2Na$
						217.1954	$C_{14}H_{26}Na$
						203.1792	$C_{13}H_{24}Na$
						187.1119	$C_{11}H_{16}ONa$
						177.1638	$C_{11}H_{22}Na$
	25.8	329.2481	$C_{22}H_{33}O_2$	38	7	/	/
	25.1	301.2143	$C_{18}H_{30}O_2Na$	61	4	/	/
Pan216 R2	21.8	**294.0532**	$C_{12}H_{12}N_3O_4S$	0	8	259.0844	? $C_{12}H_8NO_4$
						230.0451	

[a] bold mass was further processed by MS/MS

DISCUSSION

The aim of this pilot study was to develop the tools and procedures required to produce, isolate, and analyze secondary metabolites from Planctomycetes. We employed different marine and limnic Planctomycetes as a proof of principal for each step of our pipeline.

The first step was the development of cultivation conditions that support the production of secondary metabolites. To determine a suitable cultivation strategy, we took the ecology of Planctomycetes into account. It has been demonstrated by both us and others that Planctomycetes interact with phototrophs, such as cyanobacteria, in marine and limnic habitats and that the Planctomycetes can even dominate biofilms in these carbon-rich ecological niches (Bengtsson and Øvreås 2010, Bengtsson *et al.* 2010b, , Pizzetti *et al.* 2011a, Pizzetti *et al.* 2011b, Bengtsson *et al.* 2012, Jeske *et al.* 2013). Due to the slow growth of Planctomycetes, we hypothesized that their dominance in biofilms could be due to production of antibiotics, which could provide an advantage over faster-growing bacteria, such as cyanobacteria. To elucidate whether environmental cues can trigger the production of secondary metabolites, we first developed both a limnic (MM1) and a marine (MM2) Maintain Medium (Figure 1). This development is vital, because it dramatically reduces the culture-media components in acetone extracts to zero (Figure S2). Thus, the MM allows for easy detection and characterization of produced metabolites. In addition, changes in production of bioactive molecules caused by altered growth conditions could be easily monitored. Such alterations could, for example, be achieved by supplementing the MM with different carbon sources. Furthermore, ecomimetic experiments can replicate the environmental interactions of Planctomycetes and phototrophs in a chemically-defined manner, allowing for determination of environmental cues which are currently unknown. In a proof of principle study we found that *N*-acetyl-D-glucosamine (NAG), a main component of the cyanobacterial cell wall, altered the secreted secondary metabolome of both *P. limnophila* and *R. baltica* (Figure 2). This finding is not surprising, since it is well known that changes in the composition of cultivation media can affect the biosynthesis of secondary metabolites (Berdy 2005). In the past, selection of growth media composition to stimulate the expression of 'silent' secondary metabolite genes or clusters was rather random. Here we utilize the chemical characteristics of the planctomycetal ecological niche to guide alterations of the medium composition which led to the production of novel secondary metabolites. Algae and bacteria produce a multitude of different compounds and it has been shown by both our group and others that many of these molecules can be utilized by Planctomycetes (Wegner *et al.* 2012, Jeske *et al.* 2013). Thus, our Maintain Medium provides a base medium that allows for addition of many kinds of substrate combinations. Subsequent analysis of changes in the secondary metabolome can be correlated with environmental cues (substrates) present in the media. Furthermore, the addition of XAD adsorber resin allowed for easy and low solvent-volume extraction and analysis of such molecules.

After developing chemically-defined growth conditions and ecomimetic, the second step in

our pipeline is the screening for biological activity. We successfully evaluated agar plate diffusion, dual culture, and MIC assays and found all three methods very useful to screen Planctomycetes. An initial screen with the cheap and easy dual culture approach allows for determination of potential antibiotic producers, whilst the agar plate diffusion and the MIC assay allow for further purification of extracts and isolation of active components. Most importantly, we found evidence for antimicrobial activity of Planctomycetes against both Gram-negative and Gram-positive bacteria. This finding might support our hypothesis of the planctomycetal interaction with phototrophs.

The third step is the correlation of bioactivity in a crude extract with a particular HPLC peak. This step is crucial for isolation and purification of bioactive compounds, facilitating subsequent structure elucidation. We developed a comprehensive approach by combining analytical and preparative HPLC with direct fractionation of extracts into 96-well plates that can be subsequently employed for activity screening (Figure 4). While the elucidation of distinct structures is beyond the scope of this study we gained some insights into the chemical composition of planctomycetal antibiotic molecules. For example, in *P. limnophila* replicate #2, an active metabolite had a molecular formula of $C_{26}H_{51}NO_6$, which is not present in the DNP, suggesting it could be a novel compound. Although *de novo* structure elucidation is impossible with MS/MS data alone, early indications can be obtained by fragmentation ions. For example, fragments of the aforementioned compound indicate that the structure consists of a long aliphatic chain and a heteroatom-containing core structure. Pan216 replicate #2 contained an active metabolite with molecular formula of $C_{12}H_{11}N_3O_4S$, this was also not in the DNP and suggests a new natural product. UV absorption maxima at 244 and 294 nm might indicate an aromatic or heteroaromatic residue. However, Pan216 replicate #1 showed no evidence of this active metabolite. Three distinct metabolites inhibiting the growth of *B. subtilis* were also produced in Pan216 replicate #2. The active metabolites had molecular formulae of $C_{22}H_{34}O_2$, $C_{22}H_{32}O_2$, and $C_{18}H_{30}O_2$, which corresponds to 6, 7, and 4 degrees of unsaturation, respectively. Dozens of compounds are known for these molecular formulae and so we cannot identify these metabolites nor state that these metabolites are new.

Both the metabolome and the bioactivity varied strongly between the replicates of *P. limnophila* and strain Pan216, implying that the production of secondary metabolites is highly variable, responsible factors could include the inoculum, oxygenation, growth stage, formation of aggregates, as well as degradation of compounds during cultivation. Thus, this study revealed that single planctomycetal strains might be able to produce a multitude of antimicrobial secondary metabolites. Therefore, Planctomycetes do not only contain the gene clusters for secondary metabolite production, but actually produce compounds with antibacterial activity. However, structure elucidation of such bioactive molecules requires large scale cultivation of planctomycetal producer strains.

To facilitate future structure elucidation attempts, the last step of our pipeline is the cultivation of marine and limnic Planctomycetes in large scale stirring tank bioreactors. We

choose a 5 L volume in 10 L computer controlled bioreactors as this allows for easy scale-up. While establishing the protocols we noticed a disparity between the approximate doubling times determined by OD_{600} (57 h) and dry weight (47 h) for *P. limnophila,* and OD_{600} (62h) and dry weight (41 h) for *R. baltica*. Therefore, future experiments using dry weight instead of optical density might provide better correlation between growth phase and secondary metabolite production. Whilst HPLC analysis of crude extracts of two replicates of 5 liter *P. limnophila* batch cultures showed differences, the most prominent peaks were reproducible and would be the obvious candidates for structure elucidation. Once these prominent compounds have been determined, batch culture of *P. limnophila* may have to be further studied to enable isolation of the compounds which appear more variable in their production. It is possible that the life cycle switch of *P. limnophila* could cause fluctuations in the secondary metabolome (Jogler *et al.* 2011). *P. limnophila* daughter cells are flagellated and live as planktonic swimmers before developing into stalked sessile cells which are capable of dividing and forming biofilms (Jogler *et al.* 2011). If both cell types react to environmental cues in the same way remains unknown. The cultures used in our pilot study were not synchronized and thus the ratio between stalked sessile cells and flagellated swimmer cells might have varied among individual experiments. In addition, increased formation of cell aggregates might explain both the differences in production of secondary metabolites and the differences between OD_{600} and dry weight measurements. This is because aggregates give different OD_{600} values than single free-living cells. Despite such limitations, our approach will allow for the large scale production of planctomycetal compounds. For future studies, alternative reactor models and synchronization of cultures could increase reproducibility in the production of planctomycetal secondary metabolites.

In summary, we presented a pipeline for the utilization of Planctomycetes as sources of novel antibiotics. Screening of three marine and limnic planctomycetal species demonstrated discovery of antimicrobial compounds. This finding further supports our hypothesis of allelopathic interactions between phototrophs and Planctomycetes, and emphasizes the usefulness of ecomimetic experiments to stimulate the production of bioactive molecules. This work will provide the starting point for the exploration of Planctomycetes as a currently untapped source of novel bioactive molecules.

ACKNOWLEDGMENT

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SUPPLEMENTATRY MATERIAL

The Supplementary Material for this article can be found online at:
http://journal.frontiersin.org/article/10.3389/fmicb.2016.01242

Supplementary Figures

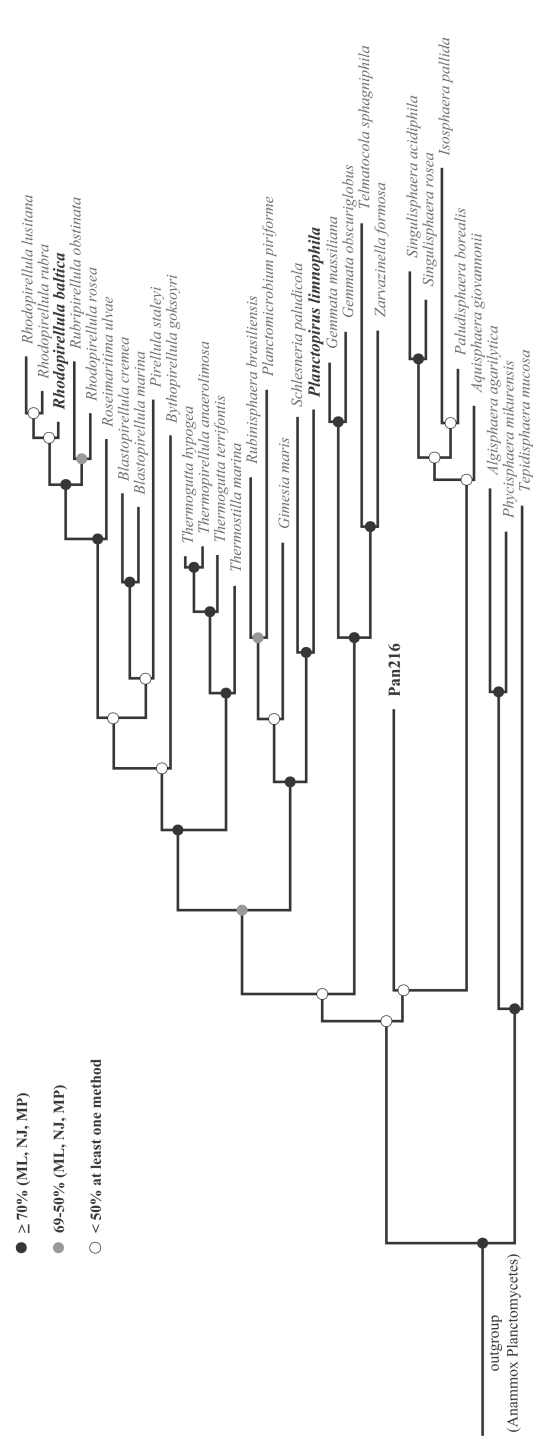

Figure S1: Phylogenetic position of strain Pan216 within the phylum Planctomycetes
The 16S rRNA gene-based tree was reconstructed under the maximum likelihood criterion with 1000 bootstrap resamplings and rooted to a cluster of sequences from anammox Planctomycetes (not shown). Strains of interest (isolate Pan216, *Planctopirus limnophila* and *Rhodopirellula baltica*) are highlighted in black and bold-faced. Validly published planctomycetal type strains are indicated by grey font and serve as matrix to pronounce the distinct phylogenetic position of strain Pan216. To estimate reliability of the tree branches, maximum pasimony and neighbor joining phylogenetic trees (not shown) were calculated (1000 bootstrap resamplings) and branch support was compared to the maximum likelihood phylogenetic tree. Black dots express bootstrap support values >70%, grey dots express support values >50% but <70% and white dots with black border express support values <50% for one method or a branch which was not supported by all three methods. Scale bar, 10% estimated sequence divergence.

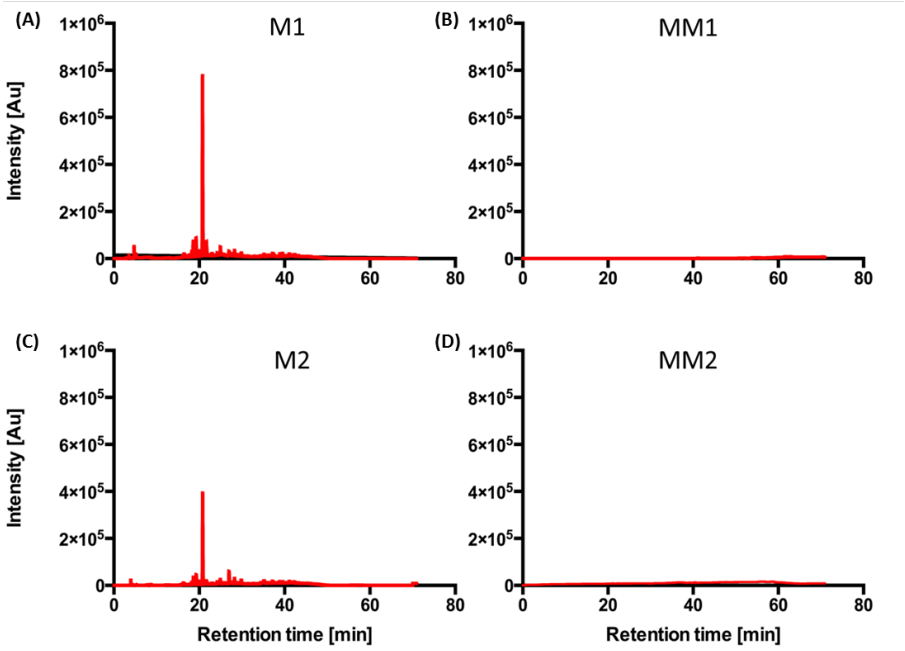

Figure S2: HPLC analysis of the marine and limnic cultivation media for Planctomycetes.
A: Full medium M1 for limnic Planctomycetes shows high signal peaks after a retention time from 3 to 10 minutes (intensity up to 8 x 10^5 Au). **B:** The chemically defined MM 1 shows no detectable signal peaks compared to its full medium M1. **C:** Full medium M2 for marine Planctomycetes shows high signal peaks after retention time from 20 to 50 minutes (intensity up to 4 x 10^5 Au). **D:** The chemically defined MM2 shows no detectable signal peaks compared to M2.

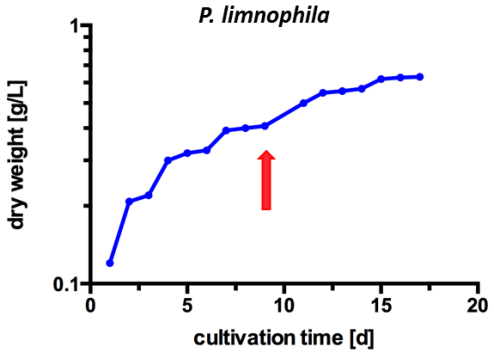

Figure S3: Dry weight determination of *P. limnophila* cells over 17 days of cultivation in 5 L fermentation with *N*-acetyl-D-glucosamine.
The dry weight of *P. limnophila* cells increased from 0.6 g/L to 2.9 g/L after 17 days cultivation. The culture was fed after 7 days with 25 mL *N*-acetyl-D-glucosamine (NAG) and 10 mL vitamin solution (red arrow). This demonstrates that a similar dry-weight could be achieved with NAG as sole carbon source if a fed-batch procedure is employed.

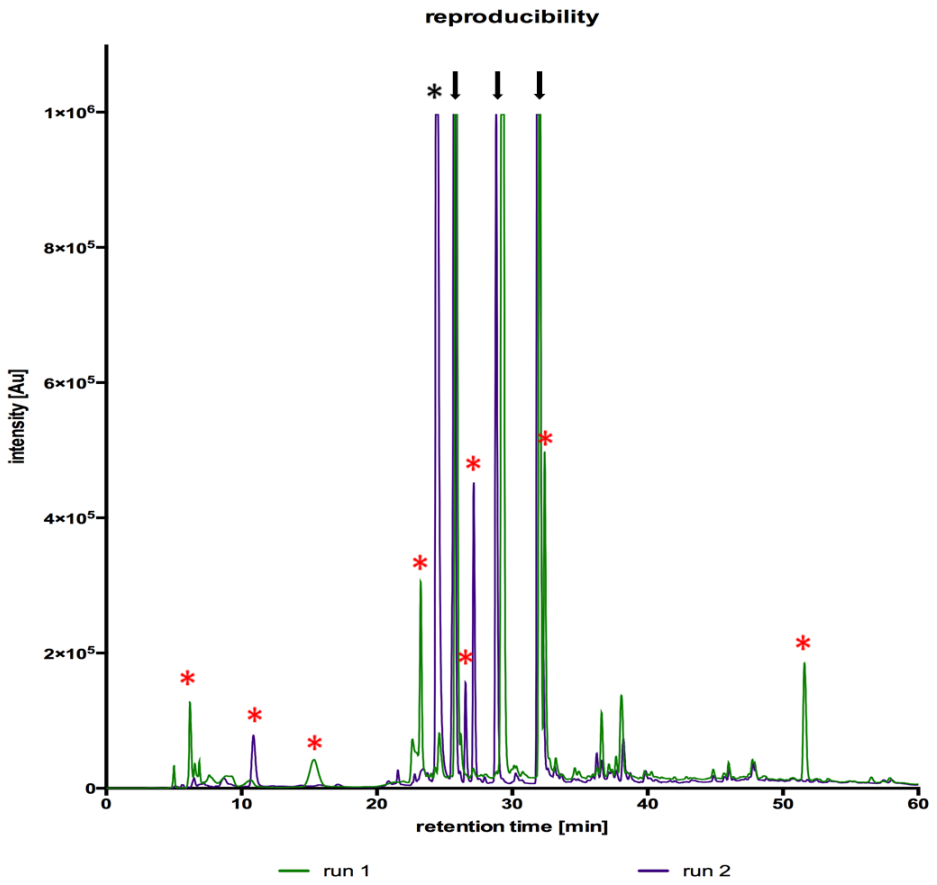

Figure S4: Reproducibility of the HPLC spectra of two distinct *P. limnophila* methanol extracts of 5 L fermentation.

In both HPLC spectra of the two distinct extracts similar signal peaks could be detected with slight shifts in retention time and intensity but also differences could be observed. Eight minor peaks (red asterisks) and one major peak (black asterisks) occurred only in one of the two replicates, while only the three major peaks (black arrows) were found in both extracts. The HPLC spectrum of the first extract (green curve) showed the first signal peak after, seven minutes and a second after 15 min but no peak at ten minutes while the HPLC spectrum of the second run (purple curve) showed the first peak at ten but not after seven or 15 min. The HPLC spectrum of the second extract revealed six signal peaks after a retention time of 25 to 32 min and an intensity of 1.56 x10^5 Au up to 10^6 Au. In the first extract an additional signal peak at 24 min was detected, the intensity of the signal peak at 25 min decreased from 10^6 Au to 9 x 10^5 Au, while the peaks at 26 and 27 min were absent. A further additional signal peak in the HPLC spectrum of the first extract was detected at 52 min that was absent in the analysis of the second extract.

SUPPLEMENTARY TABLES

Table S1: Sentinel strains for drug research of the DSMZ DZIF- collection (www.DSMZ.de)
Used organisms for anti-microbial activity screening of planctomycetal extracts in minimum inhibitory concentration assays (MIC).

	B. subtilis **DSM10**	***E. coli*** **DSM 1116**	***E. coli*** **TolC**	***M. luteus*** **DSM 1790**	***S. aureus*** **DSM 346**
OD_{600} of Aliquots	3.69	3.54	4.05	7.7	3.86
Amount cell suspension for 20 mL	54µL	56µL	49µL	26µL	52µL
Temperature	30°C	37°C	37°C	30°C	30°C
Start-OD_{600} in MIC-assay	0.01	0.01	0.01	0.01	0.01
P. limnophila **inhibition in MIC-assay**	+	-	-	+	+
R. baltica **inhibition in MIC-assay**	+	+	+	+	+
Pan216 inhibition in MIC-assay	+	-	-	+	+

Table S2: List of reference strains used for 16S ribosomal RNA gene phylogenetic tree reconstruction.
Strain designations and accession numbers were obtained from the NCBI database and the List of prokaryotic names with Standing in Nomenclature website (http://www.bacterio.net/-index.html).

Species	**Strain**	**16S rRNA gene accession number**
Algisphaera agarylitca	06SJR6-2^T	AB845176
Aquisphaera giovannonii	OJF2^T	NR_122081
Blastopirellula cremea	Pr1d^T	NR_118636
Blastopirellula marina	LHWP2^T	NR_118153
Bythopirellula goksoyri	SH 106^T	NR_029226
Gemmata massiliana	IIL30^T	JX088244
Gemmata obscuriglobus	UQM 2246^T	NR_114712
Gimesia maris	534-30^T	NR_025327
Isosphaera pallida	IS1B^T	NR_074534
Paludisphaera borealis	PX4^T	KF467528
Phycisphaera mikurensis	FYK2301M01^T	NR_074491
Pirellula staleyi	ATCC 27377^T	NR_074521
Planctomicrobium piriforme	P3^T	KP161655
Planctopirus limnophila	Mü 290^T	NR_074670
Rhodopirellula baltica	SH 1^T	NR_043384
Rhodopirellula lusitana	UC17^T	EF589351
Rhodopirellula rosea	LHWP3^T	JF748734

Rhodopirellula rubra	LF2^T	HQ845500
Roseimaritima ulvae	UC8^T	HQ845508
Rubinisphaera brasiliensis	DSM 5305^T	NR_074297
Rubripirellula obstinata	LF1^T	DQ986201
Schlesneria paludicola	MPL7^T	NR_042466
Singulisphaera acidiphila	MOB10^T	NR_102439
Singulisphaera rosea	S26^T	NR_116969
Telmatocola sphagniphila	SP2^T	NR_118328
Tepidisphaera mucosa	2842^T	KM036168
Thermogutta hypogea	SBP2^T	KC867695
Thermogutta terrifontis	R1^T	KC867694
Thermopirellula anaerolimosa	VM20-7^T	AB558583
Thermostilla marina	SVX8^T	KR872395
Zarvazinella formosa	A10^T	NR_042465
Ca. Brocadia anammoxidans	-	AF375994
Ca. Brocardia sinica	JPN1	AB565477
Ca. Jettenia asiatica	AS-1	DQ301513
Ca. Kuenenia stuttgartiensis	-	CT573071
Ca. Scalindua wagneri	-	EU478692
Ca. Scalindua brodae	-	AY257181

Stieleriacines, the First Planctomycetal Secondary Metabolites Isolated From *Stieleria maiorica* gen. nov., sp. nov.

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ABSTRACT

Bacteria belonging to the conspicuous phylum of Planctomycetes occur ubiquitously in the environment. They divide independently from FtsZ, mostly through asymmetric budding, possess a complex life cycle and contain large genomes. Despite their reported slow growth Planctomycetes can account for up to 50% of the biofilm-forming bacterial population on nutrient rich habitats such as algal surfaces. This is unexpected, since other faster-growing heterotrophs tend to colonize similar ecological niches. Despite their ecologically importance and their unexpected fitness, little is known whether Planctomycetes use secondary metabolites to successfully occupy these niches. Here we describe the isolation and elucidate the structure of the first secondary metabolites from Planctomycetes named stieleriacines A-C. These are yet unknown *N*-acyl tyrosine derivatives produced by strain Mal15^T, a novel marine Planctomycete, for which the name *Stieleria* gen. nov. is proposed. Structure elucidation of stieleriacines was performed via a combination of spectroscopic analyses, two-dimensional nuclear magnetic resonance and high resolution electron spray ionization mass spectroscopy data. Employing bioactivity-guided high-performance liquid chromatography, we could assign a moderate antibiotic activity to the stieleriacines, and limited tyrosinase activity. Moreover, the main compound stieleriacine A_1 was shown to serve as inter- and intra-species signal mediator thus being a yet unknown quorum-sensing molecule.

Taken together, we report of new *N*-acyl tyrosine derivatives with novel bioactive properties produced during laboratory cultivation of Mal15^T. Our findings might have implications for further research on bioactive molecules of Planctomycetes with potential biomedical and industrial value.

INTRODUCTION

To understand the phenotypic behavior of organisms the comprehension of their metabolism is crucial. The intermediates of metabolic reactions are divided into primary and secondary metabolites (Doelle 1975). Primary metabolites like sugars, proteins and amino acids are pivotal for the survival of the organism. Whereas secondary metabolites (SM) are not deemed necessary for survival but provide competitive advantages like through suppressed growth of neighboring species or more efficient foraging (Drew and Demain 1977). Microbial metabolites represent an amazingly diverse array of chemistry with antibiotics being the most studied SM throughout history (Hay and Fenical 1996). However, within the field of industrial microbiology SM are also known for their use as drugs including anti tumor or anti inflammatory agents, pesticides, food additives and dyes (Vaishnav and Demain 2011). The predisposition to produce these compounds is distributed in all microbial taxa (Vining 1992, Berdy 2005). However, most potent antibiotic producers are characterized by large genomes with often more than 8 Mb and complex life styles, involving for example differentiation processes (Müller and Wink 2014, Tracanna *et al.* 2017). Accordingly, in order to set priorities to find potentially talented antibiotic producers it is necessary to couple genomic information to ecological and functional data (Tracanna *et al.* 2017).

Members of the ubiquitously dwelling Planctomycetes represent an unusual group of bacteria comprising several exceptional traits (Fuerst and Sagulenko 2011, Jogler *et al.* 2012, Devos *et al.* 2013, van Niftrik 2013). They divide mostly through asymmetric budding in a FtsZ independent manner and possess a complex life cycle, switching between sessile and planktonic cells (Tekniepe *et al.* 1981, Hirsch and Müller 1985, Jogler *et al.* 2012). Moreover, they comprise large genomes up to 9 Mb and an unusual cell plan a compartmentalized cell plan (Fuerst and Webb 1991, Jeske *et al.* 2013, van Niftrik 2013, Kohn *et al.* 2016, Boedeker *et al.* 2017). While some Planctomycetes appear as free living cells in soils but also freshwater and marine habitats, others live associated with eukaryotic organisms like sponges, diatoms, cyanobacteria and micro or macro algae, where they frequently form biofilms (DeLong *et al.* 1993, Fuerst 1995, Fuerst *et al.* 1997, Arrigo 2005, Buckley *et al.* 2006, Bengtsson and Øvreås 2010, Pizzetti *et al.* 2011a). Nevertheless, they have been unnoticed in environmental samples for a long time, even though they are assumed to play critical roles in global nitrogen and carbon cycles (Arrigo 2005, Kartal *et al.* 2013). Furthermore, the biotechnological application of planctomycetal enzymes such as sulfatases as biocatalysts was demonstrated (Wallner *et al.* 2005, Gadler and Faber 2007). Overall, in recent years it became progressively obvious that these bacteria have increasing relevance in several areas of research, such as microbial ecology and oceanography (Bengtsson *et al.* 2010a, Fuerst and Sagulenko 2011, Lage and Bondoso 2011, Pizzetti *et al.* 2011a). Employing comparative genomics, it was shown that their life cycle is associated with a complex signal transduction repertoire (Jogler *et al.* 2012), hence probably involves the production of bioactive molecules.

Accordingly, Planctomycetes were anticipated to have impact on antibiotics research (Jeske *et al.* 2013, Graca *et al.* 2016, Jeske *et al.* 2016). This together makes Planctomycetes not only environmentally important but also of biotechnological and general interest.
From a phylogenetic point of view, the phylum Planctomycetes is heavily under-sampled as too few representatives of this phylum are taxonomically characterized in detail (Fuerst and Sagulenko 2011, Kohn *et al.* 2016). To date only 11 completely closed genome sequences are available through NCBI GenBank and only 31 planctomycetal species were obtained as axenic cultures (Kohn *et al.* 2016). Therefore, in this study Planctomycetes of the aerobic strain Mal15^T were isolated from seawater sediment in El Arenal, on the island Mallorca, Spain and investigated for their potential as antibiotic producers. A polyphasic taxonomy approach places the novel strain in a new genus, for which the name *Stieleria* gen. nov. is proposed. Strain designations for Mal15^T is *Stieleria maiorica* sp. nov. (DSM100215= LMG 29790), being the type species of the genus.
Employing the previously described isolation tools for antibiotic compounds from Planctomycetes (Jeske *et al.* 2016) we were able to identify five compounds with antibiotic activity and elucidated their structure using NMR and HRESIMS technology. The isolated *N*-acyl tyrosine derivatives stieleriacines A-C resembled those isolated from the marine bacterium *Thalassotalea* sp. PP2-459 (Deering *et al.* 2016). Therefore, we tested the stieleriacines for their inhibition ability of tyrosinase. To reach an either antibiotic or tyrosinase inhibition effect rather high concentrations of stieleriacines are needed. However, the ultimate role of antibiotics produced by bacteria is not conclusively resolved. First transcriptome studies of pathogenic bacteria to sub-inhibitory antibiotic concentrations demonstrated an effect on the expression of genes related to stress response, colonization, virulence, motility or biofilm formation (Yim *et al.* 2007, Skindersoe *et al.* 2008, Richards and Melander 2009, Romero *et al.* 2011). This observation has prompted the idea that antibiotics might actually act as signal molecules in natural environments, facilitating interactions within microbial communities (Davies 2006). For that reason, the main compound stieleriacine A_1 was also investigated as possible quorum-sensing molecule.

MATERIAL and METHODS

Sample Collection and Preparation

Seawater sediment samples were collected from Spain, Mallorca, El Arenal, between Balneario 4 and 5 (39° 30' 45.2'' N, 2° 44' 49.1'' E) on September 23th, 2014. Samples were collected in sterile polypropylene 50 mL tubes, transferred to the laboratory, homogenized and processed.

Culture and Isolation Conditions

Cultivation medium M1H NAG ASW was prepared with 250 mL/L double concentrated artificial sea water (46.94 g/L NaCl, 7.84 g/L Na_2SO_4, 21.28 g/L $MgCl_2 \cdot 6\ H_2O$, 2.86 g/L $CaCl_2 \cdot 2\ H_2O$, 0.384 g/L $NaHCO_3$, 1.384 g/L KCl, 0.192 g/L KBr, 0.052 g/L H_3BO_3, 0.08 g/L $SrCl_2 \cdot 6\ H_2O$, and 0.006 g/L NaF), supplemented with 0.25 g/L peptone (Bacto™), 0.25 g/L yeast extract (Bacto™), 20 mL/L mineral salts solution, 20 mL/L 5% (w/v) NAG solution, 10 mL/L 2.5% (w/v) glucose and 5 mL/L vitamin solution (double concentrated), 1 mL/L trace element solution [1500 mg/L $N(CH_2COONa)_3 \cdot H_2O$, 500 mg/L $MnSO_4 \cdot H_2O$, 100 mg/L $FeSO_4 \cdot 7H_2O$, 100 mg/L $Co(NO_3)_2 \cdot 6H_2O$, 100 mg/L $ZnCl_2$, 50 mg/L $NiCl_2 \cdot 6H_2O$, 50 mg/L H_2SeO_3, 10 mg/L $CuSO_4 \cdot 5\ H_2O$, 10 mg/L $AlK(SO_4)_2 \cdot 12H_2O$, 10 mg/L H_3BO_3, 10 mg/L $NaMoO_4 \cdot 2H_2O$, 10 mg/L $Na_2WO_4 \cdot 2H_2O$], 1 mL/L of 100 mg/mL carbenicillin and 20 mg/mL cycloheximide stock solutions, respectively and buffered with 10 mM HEPES (Serva) at pH 8.0. Solid medium was prepared with three times washed (with double distilled H_2O) 12 g/L agar (Bacto™) and cooled to 55°C prior to the addition of heat sensitive solutions. Both, mineral salt solution and double concentrated vitamin solution were prepared according to DSMZ medium 621, while metal salts solution consisted of 250 mg/L natrium-EDTA, 1095 mg/L $ZnSO_4 \cdot 7\ H_2O$, 500 mg/L $FeSO_4 \cdot 7\ H_2O$, 154 mg/L $MnSO_4 \cdot H_2O$, 39.5 mg/L $CuSO_4 \cdot 7\ H_2O$, 20.3 mg/L $CoCl_2 \cdot 6H_2O$ and 17.7 mg/L $Na_2B_4O_7 \cdot 10\ H_2O$ of which 50 mL were added per liter of mineral salt solution.

For initial bacterial isolation, solid M1H NAG ASW medium was supplemented with 100 µL of ampicillin stock solution (50 mg/mL) and cycloheximide stock solution (20 mg/mL), dried for 30 min and inoculated with 100 µL homogenized sample material per plate in a $10\text{-}10^{-2}$ dilution series and incubated at 20°C in the dark until colony formation became visible. Single colonies were inoculated on fresh solid medium with respective antibiotics. Pure cultures were cryopreserved in M1H NAG ASW medium supplemented with 50% glycerol or 5% DMSO and stored at -80 °C.

Light Microscopy

Phase contrast (Phaco) and differential interference contrast (DIC) analysis were performed employing a Nikon Eclipse Ti inverted microscope and a Nikon DS-Ri2 camera. Specimens were immobilized in MatTek glass bottom dishes (35mm, No. 1.5) employing a 1% agarose cushion. Images were analyzed using the Nikon NIS-Elements software (Version 4.3).

Electron Microscopy

For field emission scanning electron microscopy (FESEM) bacteria were fixed in 1% formaldehyde in HEPES buffer (3 mM HEPES, 0.3 mM $CaCl_2$, 0.3 mM $MgCl_2$, 2.7 mM sucrose, pH 6.9) for 1 h on ice and washed one time employing the same buffer. Cover slips with a diameter of 12 mm were coated with a poly–L–lysine solution (Sigma-Aldrich) for 10 min, washed in distilled water and air–dried. 50 µL of the fixed bacteria solution was placed on a cover slip and allowed to settle for 10 min. Cover slips were then fixed in 1% glutaraldehyde in TE buffer (20 mM TRIS, 1 mM EDTA, pH 6.9) for 5 min at room temperature and subsequently washed twice with TE-buffer before dehydrating in a graded series of acetone (10, 30, 50, 70, 90, 100%) on ice for 10 min at each concentration. Samples from the 100% acetone step were brought to room temperature before placing them in fresh 100% acetone. Samples were then subjected to critical–point drying with liquid CO_2 (CPD 300, Leica). Dried samples were covered with a gold/palladium (80/20) film by sputter coating (SCD 500, Bal–Tec) before examination in a field emission scanning electron microscope (Zeiss Merlin) using the Everhart Thornley HESE2–detector and the inlens SE-detector in a 25:75 ratio at an acceleration voltage of 5 kV.

Physiological Tests

Physiological tests such as pH and temperature tolerance were performed in liquid medium M1H NAG ASW. Temperature optima were determined by optical density measurements of growing cultures at 600 nm (OD_{600nm}). Strains were inoculated 1:10 from early stationary phase cultures in glass tubes and incubated under constant agitation in temperature controlled shakers (for exact temperatures tested, compare Figure 3). Measurements were performed in triplicates and each tube served as its own blank prior to inoculation. Resulting growth curves were analyzed by plotting change of OD_{600nm} during exponential growth phases (slope values), of each individual temperature against temperature values in °C.

To determine the pH optimum, M1H NAG ASW medium was buffered to pH values of 5.0, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0 and 10.0 using 100 mM MES, HEPES, HEPPS and CHES buffers, corresponding to their individual buffer range. OD_{600nm} was determined in glass tubes, incubated at 28°C, with three replicates as measure of growth. Growth was detected by monitoring the optical density at OD_{600nm} using a Photometer Ultrospec II (LKB Biochrom). Catalase activity was determined by bubble formation with fresh 3% H_2O_2 solution. Cytochrom oxidase activity was determined using Bactident® Oxidase test stripes (Merck Millipore) following the manufacturer's instructions. Substrate utilization of the isolated

strains was investigated using the Biolog GN2 MicroLog™ test panel for Gram-negative bacteria. Sterile glass tubes were prepared in duplicates with a basic medium mixture containing 15.7 mL IF-0a inoculation fluid (Biolog), 160 µL of 1 M HEPES buffer (pH 8.0) and 80 µL double concentrated vitamin solution. Tubes were inoculated with bacterial colony material from exponentially growing cultures to a turbidity of 54-56%. Two individual plates per strain were evaluated. To enable the comparison of the derived data, the data of each single experiment were normalized to 100. Only values corresponding to > 25% utilization were considered as positive. The heat map graphic was obtained in the R environment (RCoreTeam 2015) by using the heatmap.2() function of the gplots package. Enzymatic activities were tested using the API®ZYM method (bioMérieux) according to the manufactures protocol.

Molecular Identification and Phylogenetic Analysis

Novel isolates were identified by direct sequencing of the 16S rRNA gene after amplification with the optimized universal primers 8f (5'–AGA GTT TGA TCM TGG CTC AG–3') and 1492r (5'–GGY TAC CTT GTT ACG ACT T–3') modified from (Lane 1991). PCR reactions were performed directly on single colonies for identification or liquid cultures to check for purity, using the *Taq* DNA Polymerase Kit (Qiagen) with one reaction of 25 µL containing 11µL PCR–grade H_2O, 2.5 µL 10x CoralLoad buffer, 2.5 µL Q-Solution, 0.5 µL dNTPs (10 mM each), 1 µL sterile bovine serum albumin solution (20 mg/mL), 0.5 µl $MgCl_2$ solution (25 mM), 0.125 µL *Taq*–Polymerase (1U/µL) and 1 µL of each primer (10 pmol). The employed protocol consisted of two steps, the first step with an initial denaturation at 94°C, 5 min, 10 cycles of denaturation at 94°C, 30 sec, annealing at 59°C, 30 sec, elongation at 72°C, 1 min, followed by the second step with 20 cycles denaturation at 94°C, 30 sec, annealing at 54°C, 30 sec, elongation at 72°C, 1 min and a final elongation step at 72°C, 7 min. All PCRs were carried out in an Applied Biosystems® Veriti® thermal cycler (Thermo Fisher Scientific) and PCR products were stored at 4°C until Sanger sequencing.

Near full length 16S sequences were generated by assembly of the resulting sequences with the ContigExpress application of the Vector NTI® Advance 10 software (Thermo Fisher Scientific).

Alignment of near full length 16S rRNA sequences was performed using the SINA web aligner (Pruesse *et al.* 2012), corrected manually and used for phylogenetic tree reconstruction. Tree reconstruction was performed with the ARB software package (Ludwig *et al.* 2004) using the Maximum Likelihood RAxML module and rate distribution model GTR GAMMA running the rapid bootstrap analysis algorithm, the Neighbour Joining tool with Felsenstein correction for DNA and Maximum Parsimony method employing the Phylip DNAPARS module. Bootstrap values for all three methods were computed with 1,000 resamplings including the *E. coli* 16S rRNA gene positions 101- 1435. The analysis involved 36 nucleotide sequences of described planctomycetal type strains with anammox

Planctomycetes as outgroup (compare Table S3). 16S rRNA gene identity values of novel isolates and related type strains were calculated using neighbor joining clustering of the ARB package.

Sequencing and Gene Content Analysis

Genomic DNA of strain Mal15^T was extracted and purified with the Genomic-tip 20/G kit (Qiagen, Venlo, The Netherlands). A paired-end sequencing library was prepared with the TruSeq DNA PCR-Free LT Library Preparation Kit (Illumina, San Diego, USA) according to the manufacturer`s instructions given in TruSeq DNA PCR-Free Library Prep Reference Guide`s Low Sample (LS) Protocol for 550 bp libraries. The readily prepared sample was quantified with the NEBNext Library Quant Kit for Illumina (New England Biolabs, Ipswich, USA). An additional mate-pair sequencing library was made with the Nextera Mate Pair Library Prep Kit (Illumina) following the manufacturer`s Nextera Mate Pair Library Prep Reference Guide`s Gel-Plus protocol using agarose-gel size selection. Both libraries were sequenced on an Illumina MiSeq System employing 150 (mate-pair library) or 600 (paired-end library) cycles with MiSeq Reagent Kit v3 (Illumina).

For both types of sequencing reads, adapter clipping and read trimming was done with Trimmomatic v0.35 (Bolger *et al.* 2014). For the mate-pair data, virtual read libraries of paired-end and mate-pair reads were created previously with NxTrim v160227 (O'Connell *et al.* 2015). After adapter removal, FastQ Screen v0.4.4 (Wingett 2011) was employed to filter the data sets for contaminations and reads of low complexity were discarded with PRINSEQ lite v0.20.4 (Schmieder and Edwards 2011). Subsequently, overlapping paired-end reads were merged using FLASH v1.2.11 (Magoc and Salzberg 2011). The processed reads were assembled with SPAdes v3.7.0 (Bankevich *et al.* 2012). A scaffolding step of the pre-assembled contigs was applied by reusing the paired-end and mate-pair data with SSPACE (Boetzer *et al.* 2011) followed by an approach to close the formed gaps with Sealer (Paulino *et al.* 2015). Subsequently, a second scaffolding step was executed with MeDuSa and two unpublished, but closely related draft genomes (Bosi *et al.* 2015).

The gained consensus sequences of the strain were trimmed, circularized and adjusted to *dnaA* as the first gene. Finally, genome annotation was performed with Prokka v1.11 (Seemann 2014).

Fermentation and Isolation of Metabolites

For compound production, the nutrient richer medium M3H NAG ASW medium was used where 1.0 g/L peptone (Bacto™), 1.0 g/L yeast extract (Bacto™) were added. 600 mL Mal15^T cultures were incubated for three days at 28°C with slight agitation (80 rpm) in 2 L baffled flasks until 2% (v/v) of purified adsorbent resin XAD-16N (Rohm and Haas) were added.
In addition, also bioreactors were used. Therefore, Mal15^T cultures were grown in 5 L M3H NAG ASW under constant stirring for five days until 2% (v/v) of purified adsorbent resin XAD-16N (Rohm and Haas) were added. Afterwards, cultures in baffled flasks and bioreactors were incubated for three and five days respectively with the added XAD. In total XAD from 32 L culture were isolated and further processed.
As previously described in Jeske *et al.* 2016 the XAD-16N was collected by filtration through an analysis sieve and transferred into an extraction flask. 1/3 of the initial culture volume acetone were added to the XAD resin and incubated in darkness for two hours at room temperature. To remove the XAD resin the eluate was filtered (Macherey and Nagel 615 1/4, pore size 4-12 µm) after incubation. Employing a rotary evaporator (Hei VAP-Precision Heidolph) the acetone was removed to yield a solid residue (36°C; at 130 rpm; 24 mbar).
The combined crude extract was filtered with water / methanol (1:1) using a Strata-X 33 mm, Polymeric Reverse Phase Solid-Phase cartridge (Phenomenex, Aschaffenburg, Germany). The filtrate was disposed and the solid-phase eluted with acetone and hexane yielding 308.25 mg crude product. The crude product was fractionated using preparative RP-HPLC (PLC 2020, Gilson, Middleton, WI, USA). A Kromasil C18 column (250 x 20 mm, 7 µm (MZ-Analysetechnik, Mainz, Germany)) served as stationary phase. Deionized water (Milli-Q, Millipore, Schwalbach, Germany) with 0.1% formic acid as solvent A, and acetonitrile with 0.1% formic acid as solvent B, were used for mobile phase. The used elution gradient was 40% B for 10 min, increased to 50% in 3 min, and a gradient from 50–70% in 60 min, thereafter 100% B for 10 min. UV detection was carried out at 215 and 310 nm. Fractions were collected and combined yielding to five pure compounds. A_1 (**1**) (6.3 mg) was obtained at a retention time (t_R)= 32.5 min, B_1 (**2**) (2 mg) at t_R = 34 min, C (**3**) (0.8 mg) at t_R = 36 min, A_2 (**4**) (4.9 mg) at t_R = 39.5 min, B_2 (**5**) (3.5 mg) at t_R = 41.1 min (Figure 6).

Structure Elucidation

HRESIMS mass spectra were measured with an Agilent 1200 series HPLC-UV system in combination with an ESI-TOF-MS (Maxis, Bruker) [column 2.1 x 50 mm, 1.7 µm, C_{18} Acquity UPLC BEH (Waters), solvent A: H_2O + 0.1% formic acid, solvent B: ACN + 0.1% formic acid, gradient: 5% B for 0.5 min increasing to 100% B in 19.5 min, maintaining 100% B for another 5 min, R_F = 0.6 mL/min, UV detection 200– 600 nm]. NMR spectra were recorded on Bruker Avance III 500 MHz spectrometer with a BBFO(plus) SmartProbe (^{1}H 500 MHz, ^{13}C 126 MHz), and a Bruker Avance III 700 MHz spectrometer with a 5 mm TCI cryoprobe (^{1}H 700 MHz, ^{13}C 175 MHz, ^{15}N 71 MHz). UV spectra were recorded using a Shimadzu UV-VIS spectrophotometer UV-2450. Optical rotation was determined using a

PerkinElmer 241 polarimeter (for NMR and HRESIMS results see Supplementary Material).

Minimum Inhibitory Concentrations

Minimum Inhibitory Concentrations (MIC) were determined in a serial dilution assay in 96-well microtiter plates in YMG medium for yeasts and filamentous fungi and EBS medium (0.5% casein peptone, 0.5% glucose, 0.1% meat extract, 0.1% yeast extract, 50 mM HEPES (11.9 g/L) and pH = 7.0) for bacteria, as previously described by (Kuhnert *et al.* 2015).

Cytotoxicity Assay

The *in vitro* cytotoxicity assay was carried out against the mouse fibroblasts cell line L929 and HeLa (KB3.1) cells according to (Surup *et al.* 2015).

Tyrosinase Assay

Tyrosinase inhibition assay was performed as described by (Deering *et al.* 2016) with slight modifications. In 96-well microtiter plates 80 µL of compound, dissolved in PBS (0.1 M, pH 6.8) with 10% methanol at various concentrations, were added to 80 µL of PBS. PBS with 10% methanol also served as a negative control. Each well was given 80 µL of mushroom-tyrosinase (100 units/mL) and 40 µL of L- tyrosine (2.5 mM) was added. Each microtiter plate was accompanied by a blank with phosphate buffer, tyrosinase and the negative control. The plates were incubated at 37°C for 45 min and the absorbance measured at 490 nm using the Infinite 200 Pro multimode reader (Tecan, Switzerland). The percentage of tyrosinase inhibition was calculated as following: $[(\Delta A_{control} - \Delta A_{sample})/\Delta A_{control}] \times 100$.

Quorum-Sensing Assay

To analyze the physiological effect of stieleriacine A_1 under microfluidic conditions, a preculture of Mal15^T were inoculated 1:10 in fresh M1H NAG ASW medium and were cultivated for 72 h at 28°C at 80 rpm. Subsequently, cells were observed during their cell division cycle using the CellASIC® ONIX Microfluidic Platform (Merck Millipore). Before loading the microfluidic chamber, residues of PBS were washed with a constant flow of medium (2x 5psi, 5 min). Elastically trapped cells were than cultivated in M1H NAG ASW supplemented with stieleriacine A_1 in a as physiological concentration (0.1767 nM) assigned and hundred-fold higher concentration (17.67 nM). M1H NAG ASW was used as negative control. Cells were monitored for up to 36 h at 28°C at 2x 1psi flow rate. Images were taken every 10 min, using a Nikon Eclipse Ti with a Nikon N Plan Apochromat λ x100/1.45 oil objective and the ORCA FLASH 4.0 camera (Hamamatsu, Japan) using phase contrast microscopy. Images were processed and aligned using the NIS-elements imaging software V4.3 (Nikon). To determine different phases of cell division the cell cycle was divided into three phases. T_0 is defined as the time until the first visible sign of the formation of the daughter cell appears. T_1 is defined as the time from the first sign division until the release of the daughter cell. T_2 is defined as the time from the daughter cell release until the next visible

daughter cell formation. To determine T_0 the time of three cells in three fields of view were compared. For T_1 and T_2 three successive divisions of three different cells in three fields of view were compared.
The lyses of cells was detected over a span of 14.5 h in three fields of view. In total 1264 cells were analyzed. Hereby 428 cells showed signs of degradation.
All experiments were performed for three biological replicates.

Biofilm Formation Assay

Biofilm formation was analyzed by a modified protocol of the crystal violet assay (Frank *et al.* 2014, O'Toole and Kolter 1998). The *Phaeobacter* strains were grown in 10 mL MB medium (MB, Carl Roth) in an Erlenmeyer flask at 28°C with vigorous shaking to early exponential phase (OD_{600} ~0.4). Afterwards, cultures were diluted to an initial OD_{600} of 0.065. Following, 100 µL were transferred to a respective well of a sterile polystyrene 96-well assay plate (Corning, New York, NY, USA; Costar 3370). As needed 1.32 µL of compound A_1 in physiological (0.1767 nM) or hundred-fold (17.67 nM) solved in acetone or pure acetone were added to the wells prior to statically culturing of the plates for 24h. After incubation, medium along with planktonic cells were removed and wells were washed twice with H_2O before 200 µL 0.5% crystal violet (CV) was added to the wells and incubated at room temperature for 10 min. After staining, the CV solution was removed and each well was washed twice with H_2O to remove the residual dye and were dried overnight.
For scoring of cell attachment, the CV was extracted from the biofilm with 200 µL of 95% ethanol, of which 100 µL were transferred to a new 96-well plate before the absorbance was determined at 595 nm using the Tecan microplate reader (Infinite® 200 PRO).
For each experiment four biological replicates and two technical replicates were conducted.

RESULTS

Novel Genus Within Family *Planctomycetaceae*

Molecular Identification and Phylogenetic Analysis

Seawater sediment samples were used for the targeted isolation of novel Planctomycetes. Given that all known members of Planctomycetes show high tolerance against beta-lactam antibiotics probably due to beta-lactamase enzymes (Jeske *et al.* 2015), beta-lactam antibiotics were used as selection pressure. Obtained colonies of resistant bacteria were screened by 16S rRNA gene sequencing analysis. Phylogenetic tree reconstruction based on near full-length 16S rRNA gene sequences (Figure 1) revealed that strain Mal15^{T} belongs to the family of *Planctomycetaceae* (bootstrap support values: 70% for Maximum Likelihood, 44% for Maximum Parsimony, 62% for Neighbor Joining), sharing 94.4% sequence identity with the closest related species *Rhodopirellula baltica*^{T} (Table S3). Based on recent threshold values for 16S rRNA gene sequence comparison (Rosselló-Móra and Amann 2015), the novel strain represents a novel genus within the family *Planctomycetaceae*, for which the name *Stieleria* gen. nov. is proposed. Strain designations for Mal15^{T} is *Stieleria maiorica* sp. nov. (DSM100215= LMG 29790) being the type species of the genus.

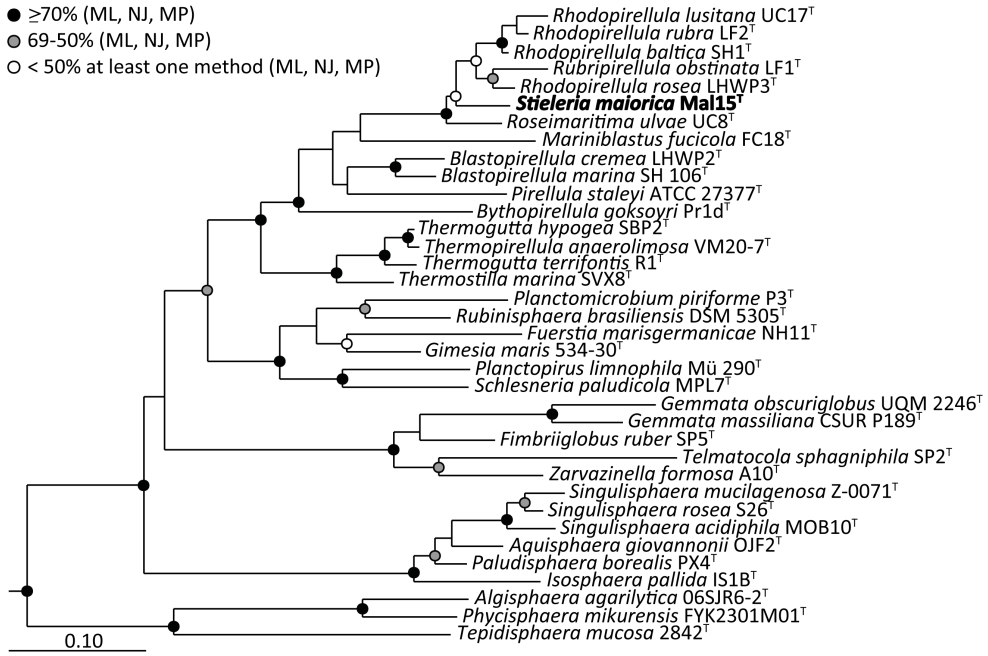


Figure 1: Phylogenetic analysis of Mal15^T

Maximum likelihood phylogenetic tree of Planctomycetes based on the 16S rRNA gene sequence (for detailed information see Table S3), while anammox Planctomycetes served as outgroup. Mal15^T (bold captured) belongs to the family Planctomycetaceae and represents a novel genus with Rhodopirellula as closest related genus. Bootstrap values based on three different tree building methods (Maximum Likelihood: ML; Neighbor Joining: NJ; Maximum Parsimony: MP). Black dots indicate support values above or equal to 70% for all three methods, while grey dots show support values of more than or equal to 50% for all three methods but less than 70%, at least for one method. White dots represent branching support of less than 50% for at least one of the employed methods. Branches that were not supported by all three methods show no dot. Scale bar indicates 10% estimated sequence divergence.

Morphological Characterization

Cells of strain Mal15^T were investigated using light microscopic and electron microscopic techniques (Figure 2), revealing round to pear shaped cells (Figure 2 A-H) with a smoothsurface (Figure 2 G-H) and form chains or rosettes (Figure 2 A-F). Cells are 1.93 ± 0.24 x 1.47 ± 0.23 µm in average (n=100 cells) (Figure 2 I). Colonies appear smooth, round and pink colored on solid medium. In liquid cultures during exponential phase cells are motile swimmers. When strain Mal15^T is grown under constant agitation (80 rpm), stirring or highly aerated, cells produce an extracellular matrix (Figure 2 G-H). Strain Mal15^T divides through polar budding (Figure 2 A-F).

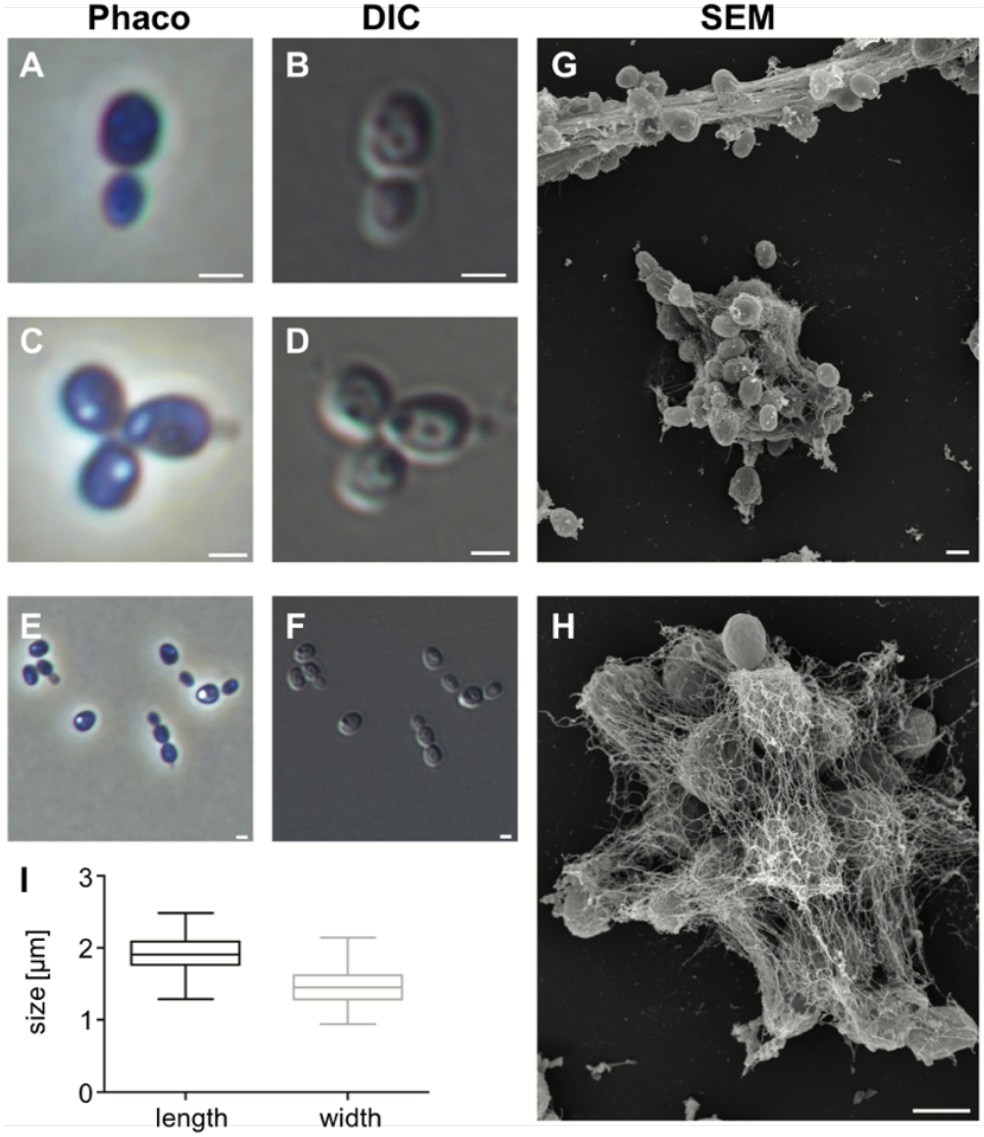


Figure 2: Morphology of strain Mal15^{T}.
A-F: Phase contrast (Phaco) and differential interference contrast (DIC) microscopy of Mal15^{T} cells. Cells are round to pear-shaped and are connected at the poles forming rosettes or chains. **G-H:** Scanning electron microscopy (SEM) revealed the existence of an extracellular matrix compound which interconnects cells in aggregates. **I:** Cell size rages between 1.93 ± 0.24 x 1.47 ± 0.23 µm (n=100 cells). Scale bar, 1 µm.

Physiological Characterization

Members of strain Mal15^T are aerobic growing planctomycetal bacteria. Compared to other Planctomycetes strain Mal15^T is fast growing, with an average division time of 4.1 h (Table 1) reaching stationary phase after approximately 60 h at optimal cultivation temperature at pH 7.5 under constant agitation (Figure 3A). Temperature optima measurements revealed a mesophilic growth profile from 10.9°C up to at least 37.3°C (Figure 3 A) with 34.7°C as optimal growth temperature (Figure 3 B). Measurements at different pH revealed growth in the range of pH 6.5 - 9 (Figure 4 A), with an optimum at pH 7.0 (Figure 4 B). Strain Mal 15^T was found to be oxidase negative and catalase positive. A conventional Gram stain was not possible for strain Mal15^T as described previously for Planctomycetes (Kohn *et al.* 2016). Employing the Biolog GN2 MicroLog™ test showed that strain Mal15^T bacteria are capable of utilizing a variety of carbon sources, listed in table S1. In particular a strong detection for *N*-acetyl D-galactosamine, *N*-acetyl-D-glucosamine, L-arabinose, D-cellobiose, L-fucose, D-fructose, D-galactose, gentiobiose, α-D-glucose, D-gluconic acid, glucuronamide, D-glucuronic acid, α-D-lactose, lactulose, D-mannose, D-melibiose, β-methyl-D-glucoside, D-raffinose, L-rhamnose, sucrose, D-trehalose, turanose and D-psicose were observed while the carbon sources acetic acid, dextrin, D-galactonic acid lacton, D-glucose-6-phosphate, α- ketoglutaric acid, maltose, D-mannitol also showed week reactions but still over 25% in both biological replicates (Table S1). Using the API®ZYM method revealed following enzyme repertoire alcaline phosphatase, esterase (C 4), esterase lipase (C 8), leucinearylamidase, valinearylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase (Table S2).

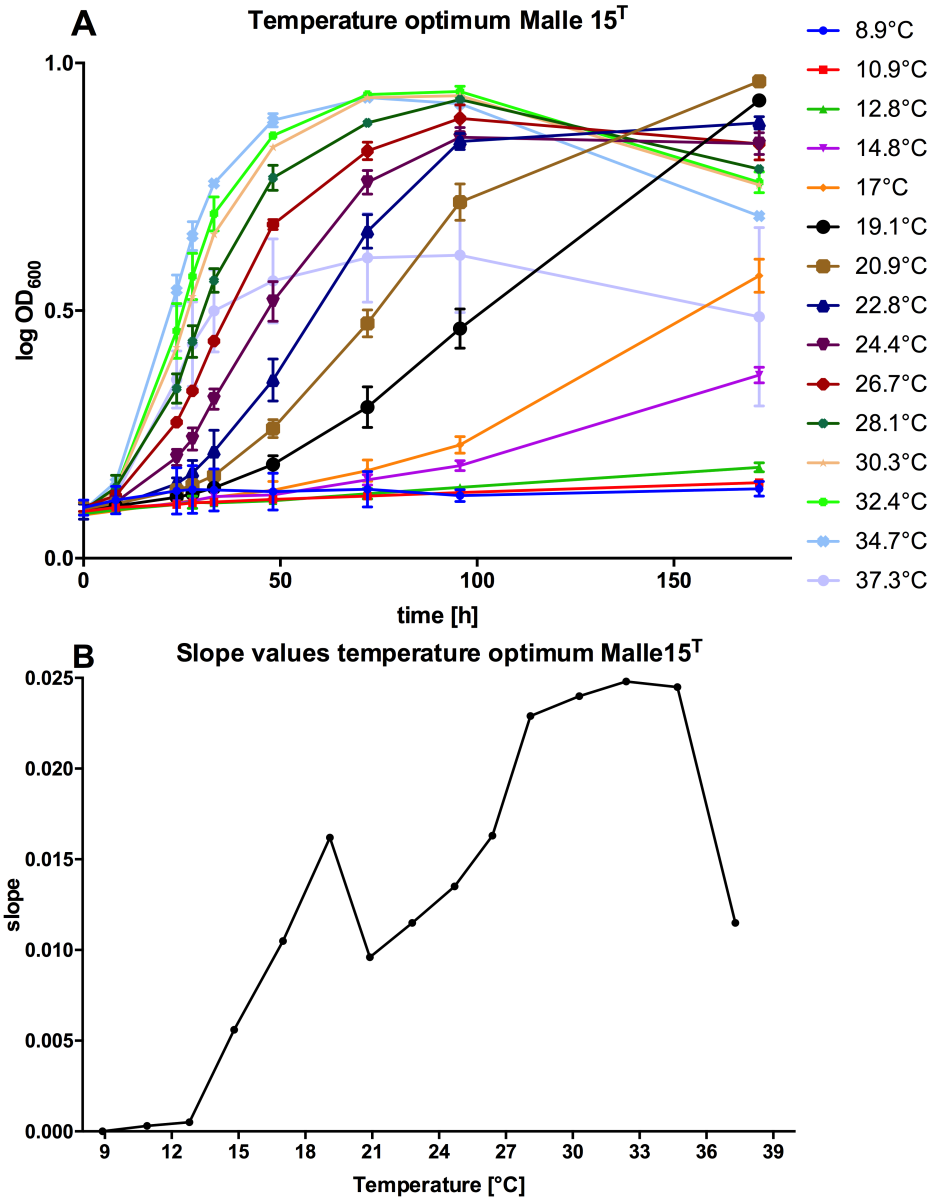


Figure 3: Temperature optimum of strain Mal15^T.
A: To determine the optimum growth temperature, optical density was measured at 600 nm (OD_{600nm}). **B:** Slope values, corresponding to change of OD_{600nm} during exponential growth phase, were plotted against the corresponding temperature value. Temperature optima was determined to be 34.7°C. Each dot represents the mean of triplicate measurements.

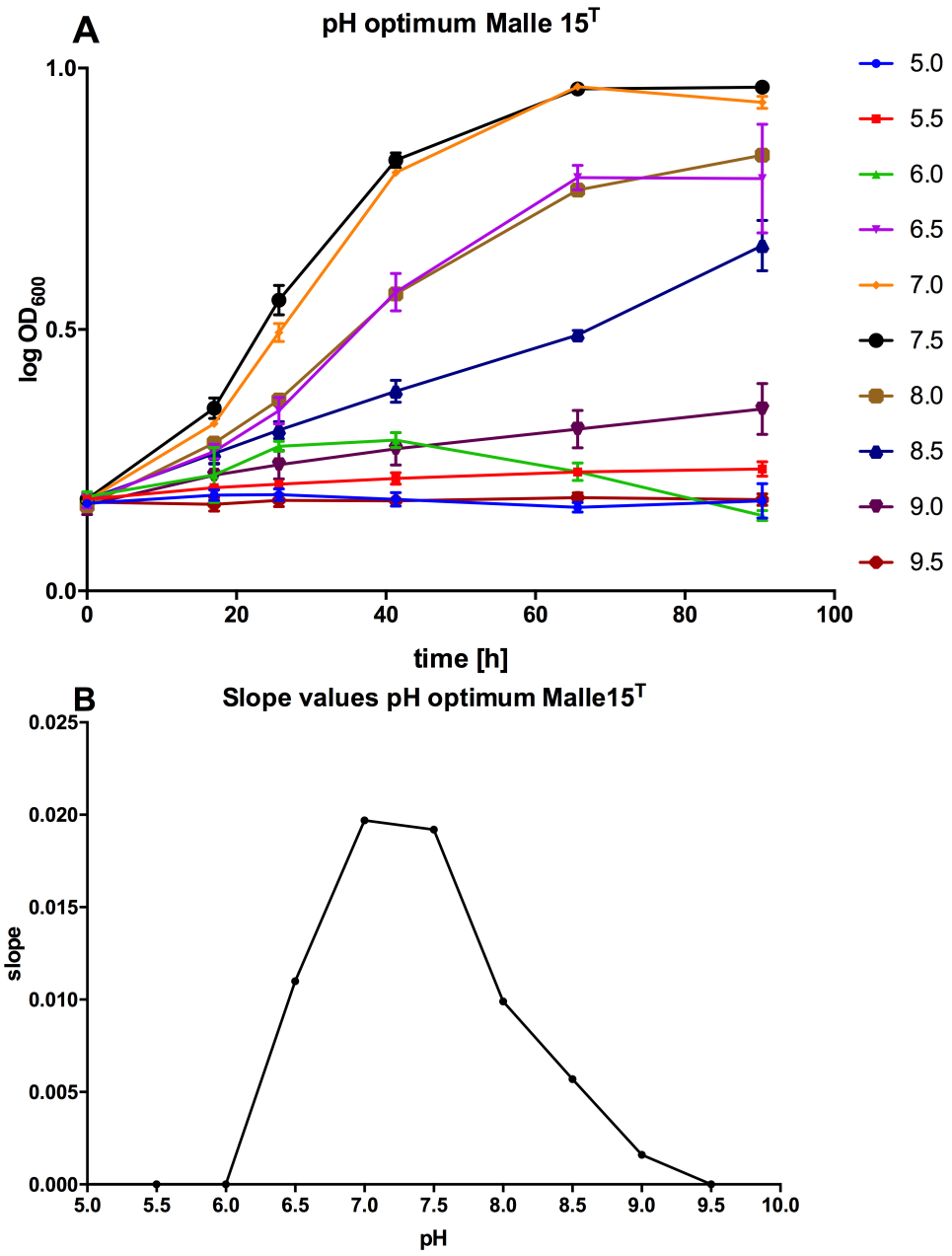
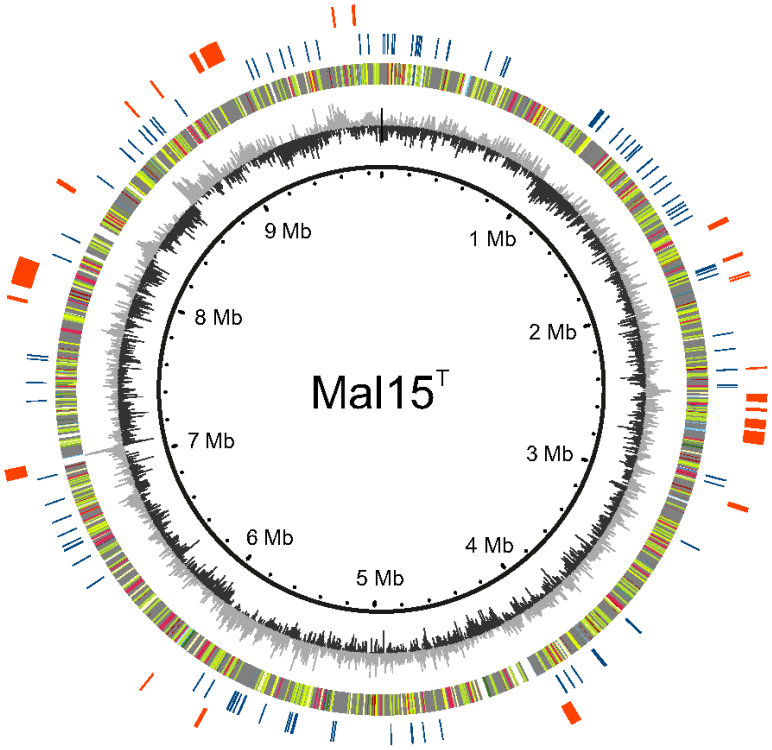


Figure 4: pH optima of strain Mal15^T.
A: To determine the optimum growth pH value, optical density was measured at 600 nm (OD_{600nm}). **B:** Slope values, corresponding to change of OD_{600nm} during exponential growth phase, were plotted against the corresponding pH. The optimum of Mal15^T was determined to be at pH 7.0. Each dot represents the mean of triplicate measurements.

Genome Analysis

The complete chromosome of strain Mal15^T comprises 9,894,293 bp, with a GC content of 59.26%. Annotation with Prokka revealed 7,016 annotated genes within the genome, of which 6,920 were identified as protein-coding genes. As known for other Planctomycetes (Fuerst and Sagulenko 2011), only for 41.89% of these genes a function could be predicted, while the remaining 2,899 genes were annotated as hypothetical proteins or proteins with unknown function. Moreover, 7 rRNA (3x 16S, 2x 23S, 2x 5S) and 81 tRNA entries were annotated. The genomic features of strain Mal15^T are summarized in Figure 5. As Planctomycetes are a known source of potential bioactive compounds (Jeske *et al.* 2013), the genome of strain Mal15^T was analyzed using antiSMASH 3.0.5 (Weber *et al.* 2015). In total, seven secondary metabolite-associated gene clusters were found. The identified genes and clusters might be related to terpenes (1), NRPS-Type 1PKS (1), Type 3 PKS (1), Type 1 PKS (1), NRPS (1) and to 'other' yet unclassified antibiotic secondary metabolite cluster (2) based on the antiSMASH 3.0.5 prediction (for more detail see chapter 3).



Cluster of Orthologous Groups (COGs)

[S] Function unknown [D] Cell cycle control, cell division, chromosome partitioning
[M] Cell wall/membrane/envelope biogenesis [N] Cell motility
[O] Post-translational modification, protein turnover, and chaperones
[T] Signal transduction mechanisms [U] Intracellular trafficking, secretion, and vesicular transport [V] Defense mechanisms [A] RNA processing and modification
[J] Translation, ribosomal structure and biogenesis [K] Transcription
[L] Replication, recombination and repair [C] Energy production and conversion
[E] Amino acid transport and metabolism [F] Nucleotide transport and metabolism
[G] Carbohydrate transport and metabolism [H] Coenzyme transport and metabolism
[I] Lipid transport and metabolism [P] Inorganic ion transport and metabolism
[Q] Secondary metabolites biosynthesis, transport, and catabolism

Core genome

Core genome of the Planctomycetes phylum

Genomic Islands

Predicted to be acquired by horizontal gene transfer

Figure 5: Circular genome plots of strain Mal15^T.
The genome of strain Mal15^T is visualized by five circles. The innermost circle is labeled with the strain name and represents the circularized genome, the plotted ticks indicate the genome size (0.2 Mb per tick). The second circle depicts the GC skew in light and dark grey. The middle circle shows the color-coded COG (Cluster of Orthologous Groups) categories of the encoded proteins. The fourth circle illustrates the core genome which was determined by the reciprocal best alignment method on three different phylogenetic levels as indicated by color. Finally, predicted genomic islands are indicated on the outermost circle.

Novel secondary metabolites derived from Mal15^T

Chemical Characterization and Structure Elucidation

The investigation of the supernatant crude extract of Mal15^T for bioactive secondary metabolites led to the isolation of five previously undescribed compounds (Figure 6 and 7). Stieleriacine A_1 (**1**) was isolated as an off-white, amorphous solid. Its molecular formula was determined by HRESIMS as $C_{22}H_{31}NO_4$, implying eight degrees of unsaturation. The ^{1}H NMR exhibited signals of three aromatic protons (δ 6.77, 7.28, 7.35), three methines (δ 6.11, 6.69, 7.17), a singlet for a methyl group (δ 2.08), methylene group proton signals (δ 1.26, 1.43, 2.18) and a triplet for a methyl group (δ 0.85). Singlets at δ 9.29 and 9.82 were attributed to an NH and OH group, respectively. The ^{13}C and distortionless enhancement by polarization transfer (DEPT) spectra show 22 carbon signals, being one carboxy, one carbonyl, three aromatic methines, three aromatic quaternary carbons, two methyls, eight methylenes, three methines, and one quaternary sp^3 hybridized carbon. ^{1}H,^{1}H correlated spectroscopy (COSY) and ^{1}H,^{13}C heteronuclear multiple bond correlation (HMBC) spectra reveal a dodecenoyl moiety with the double bond in *E* configuration between C-2' and C-3' attached via an amid bond to 8-methyl-dehydrotyrosine (Figure 8). ROE correlations support the double bond between C-2 and C-3 to be in *Z* configuration (Figure 8). The NMR data were similar, yet distinct compared to the previously reported thalassotalic acids (Deering *et al.* 2016) with differences in the length of the side chain moiety and the degree of unsaturation of the latter. Stieleriacine B_1 (**2**), an off-white amorphous powder, was identified as $C_{22}H_{33}NO_4$ by HRESIMS, indicating seven degrees of unsaturation (Figure 7). The major difference in the NMR data of **2** compared to **1** was the absence of the doublet (δ 6.11) and the doublet of triplets (δ 6.69) in the ^{1}H NMR, as well as the corresponding carbon atoms at 123.8 and 144.2 ppm, respectively. Instead a new multiplet (δ 1.55) appears corresponding to a methylene carbon signal (δ 35.3). The integration of the peak at 1.26 ppm corresponding to methylenes in the heteronuclear single quantum coherence (HSQC)-dept also shows two more protons, confirming the absence of the double bond in the side chain moiety. The carbon shift of C-3 (δ 131.7) could be retrieved from HSQC data. The shift of carbon C-2 remains unclear. Measuring of compound **2** in a different solvent, like acetone-d_6, did not bring clarity to the exact position. Likewise, to compound **1**, C-2 is also not showing any HMBC correlations. However, comparison to compounds **1** and **5** allows the suggestion that the shift of C-2 is overlapping with C-8 (δ 123.7).

Stieleriacine C (**3**), a light yellow amorphous solid, has a molecular formula of $C_{22}H_{35}NO_4$ as determined by HRESIMS, suggesting six degrees of unsaturation (Figure 7). The only noticeable difference to compound **2** in the NMR data is the appearance of a multiplet (δ 4.26) and two doublets (δ 2.87 and 2.68) in the ^{1}H NMR. The 13C NMR shows a replacement of a methine and a quaternary sp^3 hybridized carbon signal by a methylene (δ 36.1) and a methine (δ 53.9), respectively. HMBC correlations and a negative optical rotation further endorse a 3-methyl-L-tyrosine moiety.

Stieleriacine A_2 (**4**), an off-white, amorphous solid, was determined by HRESIMS to possess a molecular formula of $C_{22}H_{31}NO_4$, proposing eight degrees of unsaturation (Figure 7). Despite the absence of one carbon atom, the NMR data is fairly consistent with stieleriacine A_1 (**1**), with the only noteworthy difference in the absence of correlations between the NH proton signal with H-5 and H-9 in the rotating frame nuclear Overhauser effect spectroscopy (ROESY) spectra (Figure 8). This difference affirms the double bond between C-2 and C-3 of compound 4 to be in *E* configuration.

Stieleriacine B_2 (**5**) was isolated as an off-white, amorphous powder and could be assigned a molecular formula of $C_{22}H_{33}NO_4$ by HRESIMS, indicating seven degrees of unsaturation (Figure 7). The NMR data is in accordance with the data set of stieleriacine B_1 (**2**), with the main difference being the missing correlations of the NH proton signal with H-5 and H-9 in the ROESY spectra, which allows the conclusion that the double bond between C-2 and C-3 of compound **4** is in *E* configuration.

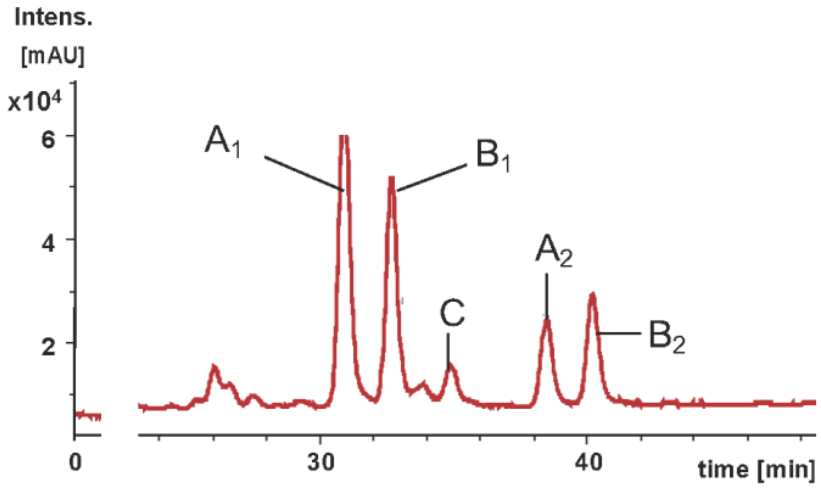


Figure 6: UV-VIS fractionation chromatogram for crude product of strain Mal15^T.
The crude product was fractionated using preparative RP-HPLC. A Kromasil C18 column served as stationary phase. Deionized water with 0.1% formic acid as solvent A, and acetonitrile with 0.1% formic acid as solvent B, were used for mobile phase. The used elution gradient was 40% B for 10 min, increased to 50% in 3 min, and a gradient from 50–70% in 60 min, thereafter 100% B for 10 min. UV detection was carried out at 215 and 310 nm. **A_1** was obtained at a retention time (t_R) = 32.5 min, B_1 at t_R = 34 min, **C** at t_R = 36 min, **A_2** at t_R = 39.5 min, **B_2** at t_R = 41.1 min

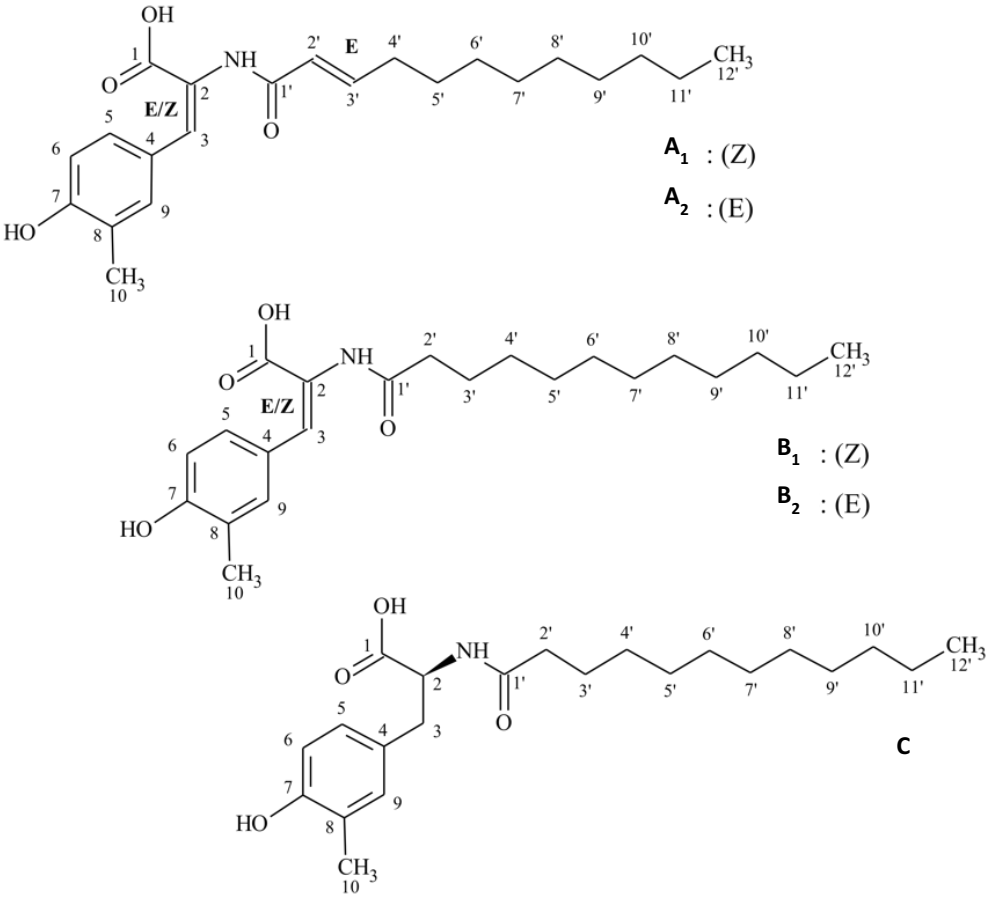


Figure 7: Structures of stieleriacines A-C.

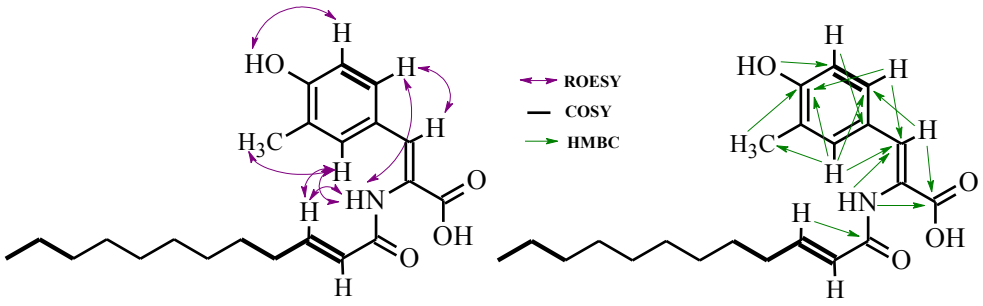


Figure 8: Key COSY, HMBC, and ROESY correlations for stieleriacine A_1 and A_2.

Biological Activities

All compounds were tested against a variety of bacteria and fungi in an antimicrobial activity assay. Stieleriacine A_1 (**1**) had weak activity against *Bacillus subtilis* (minimal inhibitory concentration (MIC) 50 µg/mL), *Mucor hiemalis* (MIC 67 µg/mL), and *Rhodotorula glutinis* (MIC 67 µg/mL). Stieleriacine B_1 (**2**) showed weak activity against *B. subtilis* (MIC 67 µg/mL) and *Micrococcus luteus* (MIC 50 µg/mL). Furthermore, stieleriacine C (**3**) also had weak activity against *B. subtilis* (MIC 67 µg/mL), *Mucor plumbeus* (MIC 50 µg/mL) and *Staphylococcus aureus* (MIC 67 µg/mL). Stieleriacine A_2 (**4**) showed weak activity against *B. subtilis* (MIC 67 µg/mL), *M. luteus* (MIC 50 µg/mL) and *S. aureus* (MIC 67 µg/mL). Likewise, stieleriacine B_2 had a weak activity against *B. subtilis* (MIC 67 µg/mL), *M. luteus* (MIC 100 µg/mL) and *S. aureus* (MIC 67 µg/mL). None of the above-mentioned compounds showed signs of cytotoxicity in a proliferation assay against the mouse fibroblast cell line L929 and HeLa KB3.1 cells. Despite their similarity to previously isolated tyrosinase inhibiting substances (Deering *et al.* 2016), stieleriacines B_1 and B_2 did not inhibit mushroom tyrosinase in the performed assay. Stieleriacines A_1 and A_2 exhibited weak inhibiting effects (IC_{50} 1.4 mM and 1.9 mM, respectively), compared to the known tyrosinase inhibitors arbutin (IC_{50} 140 µM) and kojic acid (IC_{50} 110 µM). Likewise, stieleriacine C showed weak tyrosinase inhibiting effects (IC_{50} 0.9 mM) (for more detail see Table S4).

Quorum-Sensing Assay for Planctomycetes

To analyze the physiological impact of stieleriacine A_1 on Mal15^T cells, the cell cycle was determined by time-lapse microscopy (Figure 9). The cell cycle was separated into three phases: T_0= duration of lag-phase; T_1= duration of daughter cell formation until release, T_2= duration of the gap after daughter cell release (T_1) until next daughter cell formation and T_3= duration of a complete reproduction cycle ($T_1 + T_2$). No significant difference of T_3 was observed for cells incubated with a physiological concentration (0.1767 nM) of stieleriacine A_1. Untreated cells showed a mean duration of T_1= 116.05 min and T_2= 132.71 min (T_3= 248.8 min), while treated cells showed a mean duration of T_1= 115.93 min and T_2= 137.53 min (T_3= 253.5 min) (Table 1). In Figure 9, T_0 and T_1 of untreated Mal 15^T cells (A) and cells treated with 0.1767 nM stieleriacine A_1 (B) are displayed. A significant difference was observed for T_0. Untreated cells displayed a T_0 duration of 375.6 min (6.25 h) whereas cells treated with stieleriacine A_1 required 310.0 min (5.16 h) to start division (Figure 9 A and B, Table 1). In total, T_0 duration was 65.6 min shorter in the presence of stieleriacine A_1.

Treatment of hundred-fold higher concentration of stieleriacine A_1 (17.67 nM) resulted in decreased division activity and to increased cell disruption. Statistically, 33.9% of the observed cells disintegrated after treatment with 17.67 nM stieleriacine A_1 within the time of lag-phase and two complete reproduction cycles (14.5 h =T_0 + 2x T_3) (Figure 9 C, Table 2)

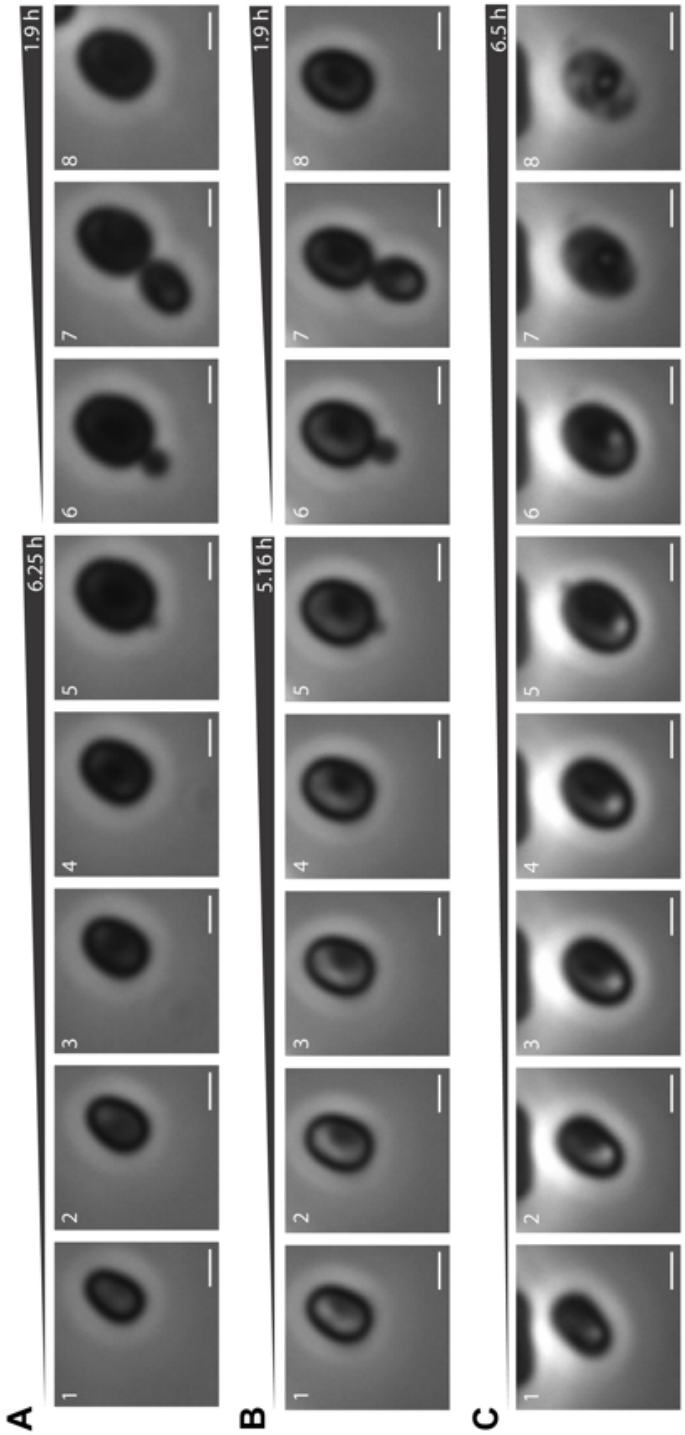


Figure 9: Time-laps series of untreated and with stieleriacine A_1 treated $Mal15^T$ cells.
A: Untreated $Mal15^T$ cells during T_0 (1-5) and T_1 (6-8). Untreated cells display a T_0 = 6.25 h and T_1=1.9 h. **B:** With 0.1767 nM stieleriacine A_1 treated $Mal15^T$ cells during T_0 (1-5) and T_1 (6-8). Treated cells display a T_0 = 5.16 h and T_1=1.9 h (for detail compare Table 1). **C:** With 17.67 nM treated $Mal15^T$ cells. Treatment results in decreased division activity and to increased cell disruption (1-8). No T_0 and T_1 could be identified (for detail compare Table 2). T_0= duration of initialization phase; T_1= duration of daughter cell formation until release. Scale bar, 1 µm.

Table 1: Duration of T_0, T_1, T_2 and T_3 for untreated and with 0.1767 nM stieleriacine A_1 treated Mal15^T cells.
T_0= duration of lag-phase; T_1= duration of daughter cell formation until release, T_2= duration of the gap after daughter cell release (T_1) until next daughter cell formation and T_3= duration of a complete reproduction cycle (T_1+T_2). Experiments were performed for three biological replicates.

Replicate	Treatment	T_0[min]	T_1[min]	T_2[min]	T_3[min]	T_3[h]
1	-	262,22	122,96	128,15	251,11	4,19
2		485,56	113,33	142,22	255,56	4,26
3		378,89	111,85	127,78	239,63	3,99
	Ø	375,56	116,05	132,72	248,77	4,15
	deviation	91,21	4,93	6,72	6,71	0,11
1	A_1	201,11	124,07	125,19	249,26	4,15
2		410,00	107,04	153,33	260,37	4,34
3		318,89	116,67	134,07	250,74	4,18
	Ø	310,00	115,93	137,53	253,46	4,22
	deviation	85,51	6,98	11,75	4,93	0,08

Table 2: Cell count of Mal15^T cells treated with 17.67 nM stieleriacine A_1 within 14.5 h.
Experiments were performed for three biological replicates. In total 1264 cells were observed. After 14.5 h (T_0 + 2x T_3), 33.9% of cells disintegrated. T_0= duration of lag- phase; **T_3**= duration of a complete reproduction cycle; FoV= field of view; disint. = disintegrated.

Replicate	FoV	intact 0 h	disint. 14.5 h	disint. [%]
1	1	154	28	18,2
	2	195	51	26,2
	3	94	23	24,5
2	1	258	117	45,3
	2	105	44	41,9
	3	56	19	33,9
3	1	51	16	31,4
	2	321	124	38,6
	3	30	6	20,0
Total		1264	428	33,9

Biofilm Formation Analysis for Roseobacter Strains

The standardized biofilm formation assay revealed a significant increase (35%, $p < 0.001$) in biofilm formation of *Phaeobacter inhibens* DSM 17395 in the presence of 17.6 nM stieleriacine A_1 (Figure 9 A). However, the same treatment of *Sulfitobacter dubius* DSM 16472^T cultures resulted in a 15% lower capacity of biofilm formation (Figure 9 A). Physiological concentrations of stieleriacine A_1 (0.176 nM) did not significantly affect the capacity to form biofilms of both *P. inhibens* and *S. dubius* (Figure 9 A). In a further approach, we tested whether stieleriacine A_1 might interact with LuxI/LuxR system as it was shown for other quorum-sensing molecules. Thus we applied the biofilm formation assay on two independent transposon mutants (Tm#3902, Tm#3163) of the exclusive LuxI/LuxR system (PGA1_c03880, (Berger *et al.* 2011)) in *P. inhibens* DSM 17395. Both Δ*luxR* transposon mutants showed almost identical absorbance values for CV at 595 nm in the biofilm formation assay. Thus, they were combined and treated as biological replicates (Figure 9 B). Controls revealed that neither the mutation of the *luxR* gene nor the presence of the transposon itself seem to influence the amount or strength of biofilm formation in the controls (Figure 9). However, results for the Δ*luxR* strains display a significant effect on the biofilm formation capacity when the Δ*luxR* cells were treated with both tested concentrations of stieleriacine A_1 (17.6 nM, 57.5% ($p < 0.001$), 0.176 nM, 17.8% ($p = 0.05$)). A significant increase of biofilm amount can be observed compared to the controls). Remarkably, the effects on biofilm formation with 17.6 nM stieleriacine A_1 are even significantly stronger (38.4%) for the Δ*luxR* mutants than for the wild type (Figure 9 B).

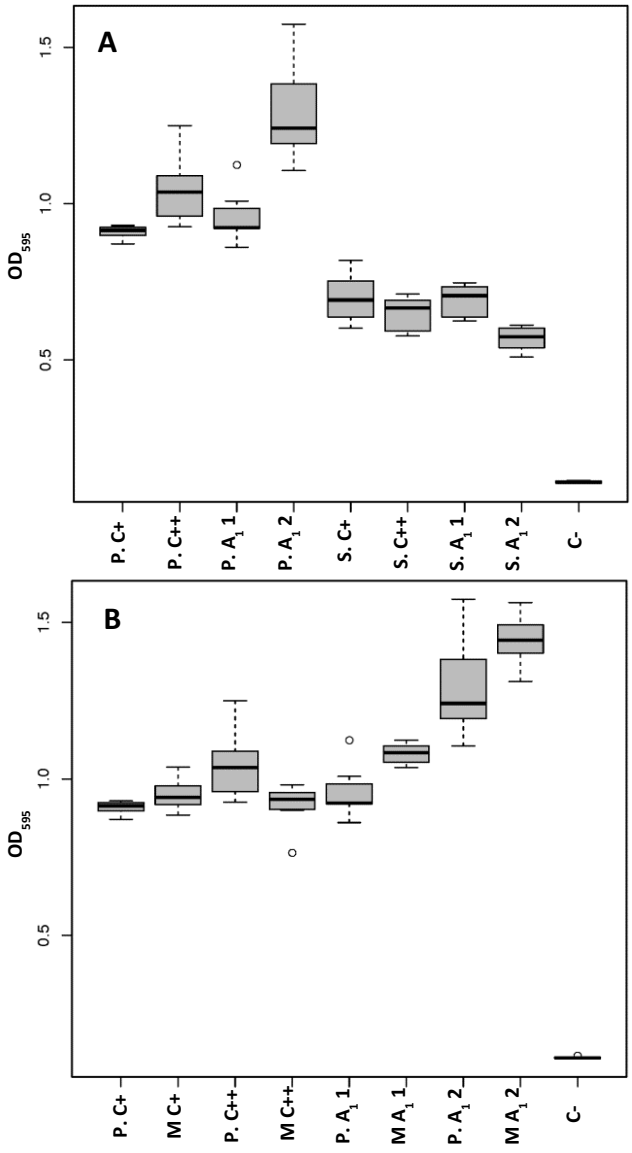


Figure 10: Box plot of biofilm formation capacity for cells treated with stieleriacine A_1.
A: Box plot of biofilm formation capacity of *P. inhibens* (P) and *S. dubius* (S).
B: Box plot of biofilm formation capacity of *P. inhibens* (P) compared to *P. inhibens* Δ*luxR* transposon mutant (M). P. C+: *P. inhibens* in MB medium; P. C++: *P. inhibens* in MB with acetone; P. A_1 1: *P. inhibens* in MB with stieleriacine A_1 0.1767 nM in acetone; P. A_1 2: *P. inhibens* in MB with stieleriacine A_1 17.67 nM in acetone; S. C+: *S. dubius* in MB medium; S. C++: *S. dubius* in MB medium with acetone; SA_1 1: *S. dubius* in MB with stieleriacine A_1 0.1767 nM in acetone; S. A_1 2: *S. dubius* in MB with stieleriacine A_1 17.67 nM in acetone; M. C+: Δ*luxR* transposon mutant in MB medium; P. C++: Δ*luxR* transposon mutant in MB with acetone; M A_1 1: Δ*luxR* transposon mutant in MB with stieleriacine A_1 0.1767 nM in acetone; M A_1 2: Δ*luxR* transposon mutant in MB with stieleriacine A_1 17.67 nM in acetone; C-: MB medium.

DISCUSSION

Stieleria maiorica

Based on 16S rRNA gene sequence comparison strain Mal15^T belongs to the family Planctomycetaceae. Sharing only 94.4% sequence identity with the closest related species *Rhodopirellula baltica*^{T} (Figure 1, Table S3) we describe strain Mal15^T as novel genus and species.

Description of *Stieleria* gen. nov.

Stieleria (Stie.le'ri.a' N.L. fem. n. *Stieleria* named in honor of Anja Heuer, maiden name Stieler, an extraordinary skilled German technician at the Leibniz Institute DSMZ, who played a key role in the cultivation of literally hundreds of novel planctomycetal strains). The round to pear shaped cells with a smooth cell surface form rosettes or short chains. Cells reproduce by polar budding. In liquid culture, they produce an extracellular matrix compound which interconnects cells in aggregates. Daughter cells are motile, while mother cells are non-motile. The life style is heterotrophic, obligatory aerobic and mesophilic. They are member of the phylum Planctomycetes, class Planctomycetacia, order Planctomycetales, family *Planctomycetaceae.* The type species is *Stieleria maiorica*.

Description of *Stieleria maiorica* sp. nov.

*Stieleria maiorica (*ma'i.ori.ca' N.L. fem. adj. *maiorica*, pertaining to the island Mallorca, Spain from which the type strain was isolated). In addition to the features described above the species exhibits following properties. Colonies on solid medium are pink colored. Cells are 1.93 ± 0.24 x 1.47 ± 0.23 µm in size. Motile daughter cells originate through budding from sessile mother cells. Gram staining delivers no clear results. The oxidase assay was negative while the catalase assay was positive. The organism is able to degrade a wide range of carbon sources. In particular strong signals were observed for *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, L-arabinose, D-cellobiose, L-fucose, D-fructose, D-galactose, gentiobiose, α-D- glucose, D-gluconic acid, glucuronamide, D-glucuronic acid, α-D-lactose, lactulose, D-mannose, D-melibiose, β-methyl-D--glucoside, D-raffinose, L-rhamnose, sucrose, D-trehalose, turanose and D-psicose while for the carbon sources acetic acid, dextrin, D-galactonic acid lacton, D-glucose-6-phosphate, α-ketoglutaric acid, maltose, D-mannitol only weak signals were detected. The enzyme repertoire includes alcaline phosphatase, esterase (C4), esterase lipase (C8), leucinearylamidase, valinearylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase. Growth occurs between pH 6.5 and 9 with an optimum at pH 7.0. The optimal growth temperature was determined to be 34.7°C but cells are able to grow in a range from 10.9°C up to at least 37.3°C. The type strain Mal15^T (DSM100215= LMG 29790) was isolated from seawater sediment.

Novel secondary metabolites derived from *Stieleria maiorica*

As Planctomycetes are presumed to be valuable sources for the discovery of novel bioactive secondary metabolites (SM) (Challinor and Bode 2015, Graca *et al.* 2016, Jeske *et al.* 2013,

Tracanna *et al.* 2017), the genome of *S. maiorica* was scanned thereupon using the newest version of antiSMASH (3.0.5) (Weber *et al.* 2015). In total, seven SM-associated gene clusters were identified (for more detail see chapter 3). As known for most Planctomycetes also *S. maiorica* was found to harbor one terpene related cluster (Jeske *et al.* 2013, Aghnatios *et al.* 2015, Graca *et al.* 2016). The remaining six clusters are related to thiotemplate modular systems (TMS) as nonribosomal peptide synthetases (NRPS) and polyketide synthetases (PKS). Interestingly, one of these clusters represents a hybrid TMS cluster related to NRPS and PKS. TMS clusters are known to be associated with the production of numerous small bioactive molecules of medical importance (Donadio *et al.* 2007). However, as described in chapter 3 most SM related genes or clusters are known to be silent under axenic laboratory cultivation conditions (van Wezel and McDowall 2011, Gomez-Escribano and Bibb 2012, Yamada *et al.* 2012) which can be also assumed for planctomycetal secondary metabolite clusters (Jeske *et al.* 2013). However, we identified five compounds with an antibiotic activity against different quality control strains of bacteria and fungi employing tools for antibiotic compound isolation and identification from Planctomycetes (Jeske *et al.* 2016). The isolated stieleriacines are to our best knowledge yet unknown *N*-acyl tyrosine (A and B) and *N*-acyl-dehydro tyrosine (C) derivatives. Interestingly, the compounds can not be assigned to any of the by antiSMASH identified clusters. Pointing towards antiSMASH does not provide the suitable algorithms to identify all SM related cluersts in Planctomycetes. The highest amount of stieleriacines could be isolated from cultures in the stationary phase which is common for most SM (Drew and Demain 1977). During this phase increased biofilm formation could be observed in culture vessels. Accordingly, *S. maiorica* is suspected to produce stieleriacines in response or at least correlated to biofilm formation. Due to the structural similarity to the recently described thalassotalic acids a role as tyrosinase inhibitor was assumed for stieleriacines. Tyrosinases are responsible for enzymatic browning accordingly are involved in ageing processes (Parvez *et al.* 2007). Planctomycetes are frequently associated with water plants or algae forming biofilms on their surface (Hempel *et al.* 2008, Bengtsson and Øvreås 2010, Matsuzawa *et al.* 2010, Bondoso *et al.* 2011, Pizzetti *et al.* 2011a, Pizzetti *et al.* 2011b). Thus, the production of a tyrosinase inhibitor would provide Planctomycetes the ability to prevent or decelerate the vegetation loss of their food source. However, only rather high amounts of stieleriacines led to an effective tyrosinase inhibition. Also, rather high concentrations of stieleriacines showed an antibiotic effect on the tested sentinel bacteria and fungi (Table S4). None of the five compounds showed signs of cytotoxicity against the tested cell lines. However, it has to be taken into account that the official quality control strains which were tested to get an overview of the inhibitory spectrum of stieleriacines are less likely to interact with Planctomycetes in nature. Thus, these strains probably do not represent the main targets for stieleriacines. Whereas marine bacteria are more likely to interact with these Planctomycetes in natural settings. *S. dubius* and *P. inhibens* are both of marine origin and are known to be associated with algae (Buchan *et al.* 2005, Frank *et al.* 2014). Stieleriacine treatment also did not affect growth of

these Roseobacter strains. However, a significant difference of the biofilm formation ability was observed, when *P. inhibens* and *S. dubius* were treated with 17.67 nM stieleriacine A_1. Interestingly, the presence of stieleriacine A_1 reduces the ability of *S. dubius* but enhances biofilm formation of *P. inhibens* (Figure 10). *P. inhibens* is known to produce a hormone presumed to promote algal growth and the antibiotic tropodithietic acid (TDA) (Wang *et al.* 2016). Planctomycetes however are insensitive to TDA. Accordingly, *P. inhibens* could improve the fitness of planctomycetal biofilm communities by providing antibiotic protection against competing bacteria and fosters their joint food source. Nothing comparable is known for *S. dubius*. Stieleriacine A_1 in different concentrations also affects the *S. maiorica* culture itself in diverging ways. Low concentrations of stieleriacine A_1 (0.1767 nM), which were ascertained to be the physiological concentration in the original cultures, reduced significantly the duration of the lag-phase (T_0) and thus might accelerate biofilm formation (Figure 9, Table 1). Whereas, a hundred-fold higher concentration (17.67 nM) lead to decreased division activity and to increased cell death (Figure 9, Table 2). These findings may allow the hypothesis that the secreted stieleriacine A_1 might act as inter- and intra-species quorum-sensing molecule. According to this hypothesis, motile Planctomycetes may sense stieleriacine A_1 that was secreted by other sessile Planctomycetes which inhabit a favorable habitat. As a consequence, the motile Planctomycetes probably switch from motile cells into sessile cells, start reproduction and thus magnify the planctomycetal biofilm. Moreover, also other bacteria that may improve the planctomycetal biofilm community might be attracted whereas bacteria that would not provide advantage to the planctomycetal community are repelled by the same molecule. According to the difference of concentration by which *S. maiorica* and *P. inhibens* are affected by stieleriacine A_1, we assume that *P. inhibens* cells are not affected until a critical number of Planctomycetes meaning a high stieleriacine A_1 concentration has been reached. This mechanism would ensure that *S. maiorica* would not be overgrown by *P. inhibens* in the first place.

Quorum-sensing (QS) is a chemical communication process that bacteria use to regulate collective behaviors (Fuqua *et al.* 1994). The fundamental steps are analogous in all known QS systems (Miller and Bassler 2001). First, so-called autoinducers are synthesized and released outside of the cells. Second, after a threshold level required for detection by neighboring cells is exceeded cognate receptors bind the autoinducer and trigger signal transduction cascades that result in population-wide changes in gene expression (Miller and Bassler 2001). In Gram-negative bacteria, the best-known autoinducers are *N*-acyl homoserine lactones (AHL) (Nealson 1977, Fuqua *et al.* 1994,). The detection and gene activation is regulated by the generally called LuxI/LuxR system or lux box (Fuqua *et al.* 1994). The lux box represents a conserved regulatory sequence in Gram-negative bacteria (Callahan and Dunlap 2000, Hao *et al.* 2010). Through structural differences AHL molecules are typically unique in that, a particular AHL molecule is detected only by the species that produces it (Fuqua and Greenberg 2002). Therefore, it is suggested that AHL QS systems predominately foster intra-species communication (for review see

(Ng and Bassler 2009)). However, as described above stieleriacine A_1 influenced not only *S. maiorica*'s behavior but also had an effect on biofilm formation of *P. inhibens*. Accordingly, stieleriacine A_1 might also function as inter-species QS mediator. However, *N*-acyl tyrosines have previously been suggested but not proven to be involved in QS (Craig *et al.* 2011). To determine if stieleriacine A_1 might act in the same mode of action that is known for AHLs, Δ*luxR* mutants of *P. inhibens* (for more detail see Supplementary Experiments) were treated with the same concentration as wild type *P. inhibens*. Surprisingly, compared to the wild type the biofilm formation ability of the Δ*luxR* strains increased even significantly more when cells were treated with stieleriacine A_1. In addition, also the lower concentration of stieleriacine A_1 was sufficient to trigger a significant increasing. Thus, instead of not being able to sense the molecule it seems as the mutant strains became even more sensitive to the compound. Since *P. inhibens* possess only one *luxR* homologue (Berger *et al.* 2011) this results point towards a mechanism that is not relaying on the lux box QS system. This assumption is supported by the fact that the *S. maiorica* genome possesses different *luxR* encoding domains but *luxI* genes do not appear to be present in its genome. This precludes accessorily the assumption that stieleriacine A_1 would target the lux box. Preventively, the utilization of stieleriacine A_1 as carbon source was excluded in the experimental settings for *S. maiorica* and *P. inhibens*. Therefore, another, yet unknown mode of action has to be assumed for stieleriacine A_1. However, this assumption requires further verification. For example, transcriptome analyses could be performed to monitor the transcriptional up or down regulation of genes in response to the treatment with stieleriacines. Moreover, the hypothesis of stieleriacines being related to biofilm formation among different species needs a comprehensive verification. For AHLs it was observed that the *luxI* gene encoding for the AHL synthase (Fuqua *et al.* 1994) is co-localized to the exopolysaccharide biosynthesis genes in *Pantonea stewartii* (von Bodman *et al.* 1998). This might be also true for the stieleriacine encoding gene. It might be in close proximity to such synthesis genes to start exopolysaccharide production and thus regulates biofilm formation processes. As QS is used by bacteria to regulate collective behaviors, disabling QS circuits has been proposed as a potential strategy to prevent bacterial pathogenicity (Cegelski *et al.* 2008, Shapiro and Wencewicz 2016). Constantly, unexpected functions of known SM are being unraveled. The chemical diversity of microbial metabolites remains a seemingly infinite source for the discovery of novel compounds that might be useful for various applications (Vaishnav and Demain 2011). However, the discovery of antibiotics seemed to be redeeming in the fight of bacteria associated diseases but antibiotic resistance poses again the same threat to public health as 80 years before (WHO 2014). Therefore, we must certainly focus on the discovery of novel compounds but maybe even more important on novel mechanisms that are not as easily overcome by pathogenic bacteria as antibiotics. In particular, at first we need to take advantage of already known molecules as well as new derivatives to be made by chemists and microbial molecular geneticists (Vaishnav and Demain 2011) but in the long-term we need to find alternatives to antibiotic treatment. Thus, even though

stieleriacines exhibit only low antibiotic properties they could be chemically modified to stronger action. However, obtained knowledge of the mode of action of a presumably novel QS system might pave the way for innovative treatment approaches of bacteria that are associated for example with biofilm formation. Moreover, the area of possible application for QS interference extends not only to medicine but also to applied domains like agronomy or aquaculture and other industrial sectors (Fitridge *et al.* 2012, Grandclement *et al.* 2016).

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SUPLEMENTARY MATERIAL

HRESIMS and NMR Results for Stieleriacines A-C

Stieleriacine A_1 (**1**)

(Z isomer); off-white, amorphous powder; UV (MeOH) λ_{max} (log ε) 200 (2.6), 314 (2.2) nm. HRESIMS m/z 374.2325 $[M+H]^+$ (calcd for $C_{22}H_{31}NO_4$, 373.2253). ^{1}H NMR (700MHz, DMSO-d_6) δ = 9.82 (1H, s, C-OH-7), 9.29 (1H, s, NH), 7.35 (1H, d, J = 1.7 Hz, H-9), 7.28 (1H, dd, J = 8.4 Hz, 1.9 Hz, H-5), 7.17 (1H, s, H-3), 6.77 (1H, d, J = 8.4 Hz, H-6), 6.69 (1H, dt, J = 15.4 Hz, 6.9 Hz, H-3'), 6.11 (1H, d, J = 15.5 Hz, H-2'), 2.18 (2H, q, J = 1.0 Hz, H-4'), 2.08 (3H, m, H-10), 1.43 (2H, tquin, J = 7.3 Hz, 1.0 Hz, H-5'), 1.26 (14H, m, H-6', 7', 8', 9', 10', 11'), 0.85 (3H, dt, J = 14.6 Hz, 7.1 Hz, H-12'). ^{13}C NMR (176MHz, DMSO-d_6) δ = 166.6 (C-1), 164.4 (C-1'), 156.9 (C-7), 144.2 (C-3'), 132.7 (C-9), 132.6 (C-3), 129.3 (C-5), 124.5 (C-4), 123.9 (C-8), 123.8 (C-2'), 123.6 (C-2), 114.6 (C-6), 31.3 (C-4'), 31.3 (C-10'), 29.0 - 28.6 (C-6', 7', 8', 9'), 27.8 (C-5'), 22.1 (C-11'), 16.0 (C-10), 14.0 (C-12').

Stieleriacine B_1 (**2**)

(Z isomer); off-white, amorphous powder; UV (MeOH) λ_{max} (log ε) 201 (1.5), 227 (1.4), 310 (1.6) nm. HRESIMS m/z 376.2490 $[M+H]^+$ (calcd for $C_{22}H_{33}NO_4$, 375.2409).
^{1}H NMR (500MHz, DMSO-d_6) δ = 9.13 (1H, br s, NH), 7.37 (1H, s, H-9), 7.25 (1H, d, J = 7.6 Hz, H-5), 7.12 (1H, s, H-3), 6.76 (1H, d, J = 8.2 Hz, H-6), 2.24 (2H, t, J = 7.3 Hz, H-2'), 2.09 (3H, s, H-10), 1.55 (2H, m, H-3'), 1.26 (16H, m, H-4', 5', 6', 7', 8', 9', 10', 11'), 0.86 (3H, t, J = 7.2 Hz, H-12'). ^{13}C NMR (126MHz, DMSO-d_6) δ = 171.7 (C-1'), 167.0 (C-1), 156.6 (C-7), 132.4 (C-9), 131.7[1] (C-3), 129.4 (C-5), 124.8 (C-4), 123.7 (C-8, 2), 114.5 (C-6), 35.3 (C-2'), 31.3 (C-10'), 29.0 - 28.7 (C-4', 5', 6', 7', 8', 9'), 25.1 (C-3'), 22.1 (C-11'), 16.0 (C-10), 13.9 (C-12').

Stieleriacine C (**3**)

Light yellow amorphous solid; $[\alpha]_D^{21}$ -28 (c = 1, CH_3OH); UV (MeOH) λ_{max} (log ε) 202 (1.8), 227 (1.3), 279 (0.8), 312 (0.2) nm. HRESIMS m/z 378.2653 $[M+H]^+$ (calcd for $C_{22}H_{35}NO_4$, 377.2566). ^{1}H NMR (700MHz, DMSO-d_6) δ = 7.86 (1H, br s, NH), 6.86 (1H, s, H-9), 6.79 (1H, dd, J = 8.2 Hz, 1.8 Hz, H-5), 6.62 (1H, d, J = 8.0 Hz, H-6), 4.26 (1H, m, H-2), 2.87 (1H, dd, J = 13.8 Hz, 5.0 Hz, H-3a), 2.68 (1H, dd, J = 13.8 Hz, 9.1 Hz, H-3b), 2.05 (3H, s, H-10), 2.02 (2H, td, J = 7.3 Hz, 2.5 Hz, H-2'), 1.39 (2H, m, H-3'), 1.22 (14H, m, H-5', 6', 7', 8', 9', 10', 11'), 1.14 (2H, m, H-4'), 0.85 (3H, t, J = 7.0 Hz, H-12'). ^{13}C NMR (176MHz, DMSO-d_6) δ = 173.3 (C-1), 171.9 (C-1'), 153.8 (C-7), 131.3 (C-9), 127.8 (C-4), 127.1 (C-5), 123.1 (C-8), 114.2 (C-6), 53.9 (C-2), 36.1 (C-3), 35.2 (C-2'), 31.3 (C-10'), 29.0 - 28.7 (C-5', 6', 7', 8', 9'), 28.5 (C-4'), 25.2 (C-3'), 22.1 (C-11'), 16.0 (C-10), 13.9 (C-12')

Stieleriacine A_2 (**4**)

(E isomer); off-white, amorphous powder; UV (MeOH) λ_{max} (log ε) 210 (1.6), 314 (1.6) nm.

[1] ^{13}C shift extracted from HSQC data.

HRESIMS m/z 374.2336 $[M+H]^+$ (calcd for $C_{22}H_{31}NO_4$, 373.2253). ^1H NMR (500MHz, DMSO-d_6) δ = 9.28 (1H, s, NH), 7.34 (1H, s, H-9), 7.28 (1H, dd, J = 8.3 Hz, 1.9 Hz, H-5), 7.17 (1H, s, H-3), 6.78 (1H, d, J = 8.4 Hz, H-6), 6.69 (1H, m, H-3'), 6.12 (1H, d, J = 15.4 Hz, H-2'), 2.18 (2H, m, H-4'), 2.09 (3H, s, H-10), 1.44 (2H, m, H-5'), 1.28 (14H, m, H-5', 6', 7', 8', 9', 10', 11'), 0.87 (3H, t, J = 6.6 Hz, H-12'). ^{13}C NMR (126MHz, DMSO-d_6) δ = 166.7 (C-1), 164.3 (C-1'), 156.8 (C-7), 144.1 (C-3'), 132.7 (C-9), 132.2 (C-3), 129.2 (C-5), 124.6 (C-4), 123.8 (C-8, 2, 2'), 114.6 (C-6), 31.3 (C-4'), 31.3 (C-10'), 29.0 - 28.6 (C-6', 7', 8', 9'), 27.8 (C-5'), 22.1 (C-11'), 16.0 (C-10), 13.9 (C-12').

Stieleriacine B_2 (**5**)

(E isomer); off-white, amorphous powder; UV (MeOH) λ_{max} (log ε) 202 (1.6), 310 (1.5) nm. HRESIMS m/z 376.2498 $[M+H]^+$ (calcd for $C_{22}H_{33}NO_4$, 375.2409). ^1H NMR (500MHz, DMSO-d_6) δ = 9.18 (1H, s, NH), 7.40 (1H, m, H-9), 7.28 (1H, dd, J = 8.3 Hz, 1.4 Hz, H-5), 7.14 (1H, s, H-3), 6.77 (1H, d, J = 8.4 Hz, H-6), 2.24 (2H, t, J = 7.3 Hz, H-2'), 2.10 (3H, s, H-10), 1.56 (2H, m, H-3'), 1.20 - 1.32 (16H, m, H-4', 5', 6', 7', 8', 9', 10', 11'), 0.85 (3H, t, J = 6.9 Hz, H-12'). ^{13}C NMR (126MHz, DMSO-d_6) δ = 171.9 (C-1'), 166.8 (C-1), 156.8 (C-7), 132.5 (C-9), 132.4 (C-3), 129.5 (C-5), 124.5 (C-4), 124.0 (C-8), 123.8 (C-2), 114.5 (C-6), 35.2 (C-2'), 31.3 (C-10'), 29.0 - 28.6 (C-4', 5', 6', 7', 8', 9'), 25.1 (C-3'), 22.1 (C-11'), 16.0 (C-10), 13.9 (C-12').

SUPPLEMETARY TABLES

Table S1: Carbon sources utilized by strain Mal15^T.
+=positive; W=weakly positive

Carbon sources	
Acetic acid	W
N-Acetyl-D-Galactosamine	+
N-Acetyl-D-Glucosamine	+
L-Arabinose	+
D-Cellobiose	+
Dextrin	W
L-Fucose	+
D-Fructose	+
D-Galactose	+
D-Galactonic acid lacton	W
Gentiobiose	+
α-D-Glucose	+
D-Glucose- 6-phosphate	W
D-Gluconic acid	+
Glucuronamide	+
D-Glucuronic acid	+
α-Ketoglutaric acid	W
α-D-Lactose	+
Lactulose	+
Maltose	W
D-Mannose	+
D-Mannitol	W
D-Melibiose	+
β-Methyl-D-Glucoside	+
D-Raffinose	+
L-Rhamnose	+
Sucrose	+
D-Trehalose	+
Turanose	+
D-Psicose	+

Table S2: Enzymatic activities of strain Mal15^T, determined with API®ZYM test.
+=positive; -=negative

Enzymatic activities	
Alcaline phosphatase	+
Esterase (C 4)	+
Esterase lipase (C 8)	+
Lipase (C 14)	-
Leucinearylamidase	+
Valinearylamidase	+
Cysteine arylamidase	-
Trypsin	-
α-chymotrypsin	-
Acid phosphatase	+
Naphthol-AS-BI-phosphohydrolase	+
α-galactosidase	-
β-galactosidase	-
β-glucuronidase	-
α-glucosidase	-
β-glucosidase	-
N-acetyl-β-glucosaminidase	-
α-mannosidase	-
α-fucosidase	-

Table S3: List of reference and out-group strains for 16S rRNA phylogenetic tree reconstruction.
To resolve the position of the novel strain Mal15^T, 16S rRNA sequences of planctomycetal type strains were included in the analysis. Anammox Planctomycetes served as outgroup (grey). Mal15^T in the phylogenetic tree (Figure 1) is displayed in bold capture.

Species	Strain	16S rRNA accession nr.
Algisphaera agarilytica	06SJR6-2^T	NR_125472
Aquisphaera giovannonii	OJF2^T	NR_122081
Bythopirellula goksoyri	Pr1d^T	NR_118636
Blastopirellula cremea	LHWP2^T	NR_118153
Blastopirellula marina	SH 106^T	NR_117648
Fimbriiglobus ruber	SP5^T	KX369544.1
Fuerstia marisgermanicae	NH11^T	CP017641
Gemmata massiliana	CSUR P189^T	JX088244
Gemmata obscuriglobus	UQM 2246^T	NR_118911
Gimesia maris	534-30^T	NR_025327
Isosphaera pallida	IS1B^T	NR_074534
Mariniblastus fucicola	FC18^T	HQ845450
Paludisphaera borealis	PX4^T	CP019082
Phycisphaera mikurensis	FYK2301M01^T	NR_074491
Pirellula staleyi	ATCC 27377^T	NR_074521
Planctomicrobium piriforme	P3^T	KP161655
Planctopirus limnophila	Mü 290^T	NR_074670
Rhodopirellula baltica	SH1^T	NR_043384
Rhodopirellula lusitana	UC17^T	NR_126199
Rhodopirellula rosea	LHWP3^T	NR_132692
Rhodopirellula rubra	LF2^T	NR_126223
Roseimaritima ulvae	UC8^T	HQ845508
Rubinisphaera brasiliensis	DSM 5305^T	NR_074297
Rubripirellula obstinata	LF1^T	DQ986201.2
Schlesneria paludicola	MPL7^T	NR_042466
Singulisphaera acidiphila	MOB10^T	NR_042662.1
Singulisphaera mucilagenosa	Z-0071^T	HM748856.1
Singulisphaera rosea	S26^T	NR_116969
Stieleria maiorica	Mal15^T	
Telmatocola sphagniphila	SP2^T	NR_118328
Thermopirellula anaerolimosa	VM20-7	AB558583
Thermogutta hypogea	SBP2^T	NR_134825.1
Thermogutta terrifontis	R1^T	NR_134826.1
Thermostilla marina	SVX8^T	KR872395.1
Tepidisphaera mucosa	2842^T	KM036168
Zavarzinella formosa	A10^T	NR_042465
Ca. Brocadia anammoxidans	-	AF375994

Ca. Brocadia fulgida	-	DQ459989.1
Ca. Brocadia sinica	JPN1	AB565477
Ca. Jettenia asiatica	AS-1	DQ301513
Ca. Jettenia caeni	KSU-1	AB057453.1
Ca. Kuenenia stuttgartiensis	-	AF375995.1
Ca. Scalindua brodae	BS 5	AY257181

Table S4: MIC results for bacteria and fungi sentinel strains treated with stieleriacines.
n.i. not indicated

	A_1 (**1**) [µg/mL]	B_1 (**2**) [µg/mL]	C (**3**) [µg/mL]	A_2 (**4**) [µg/mL]	B_2 (**5**) [µg/mL]
Bacillus subtilis DSM 10	50	67	67	67	67
Escherichia coli DSM 1116	n.i.	n.i.	n.i.	n.i.	n.i.
Mucor plumbeus MUCL49355	n.i.	n.i.	50	n.i.	n.i.
Candida tenuis MUCL29892	n.i.	n.i.	n.i.	n.i.	n.i.
Candida albicans DSM1665	n.i.	n.i.	n.i.	n.i.	n.i.
Pichia anomala DSM 6766	n.i.	n.i.	n.i.	n.i.	n.i.
Micrococcus luteus DSM 1790	n.i.	50	n.i.	50	100
Chromobacterium violaceum DSM 30191	n.i.	n.i.	n.i.	n.i.	n.i.
Pseudomonas aerogiΩnosa PA14	n.i.	n.i.	n.i.	n.i.	n.i.
Mycobacterium smegmatis ATCC 700084	n.i.	n.i.	n.i.	n.i.	n.i.
Schizosaccharomyces pombe DSM 70572	n.i.	n.i.	n.i.	n.i.	n.i.
Staphylococcus aureus DSM 346	n.i.	n.i.	67	67	67
Rhodotorula glutinis DSM 10134	67	n.i.	n.i.	n.i.	n.i.
Mucor hiemalis DSM 2656	67	n.i.	n.i.	n.i.	n.i.

SUPPLEMENTARY EXPERIMENTS

Verification of transposon mutants Tm#3163 and Tm#3902

Former experiments with EZ-Tn5 transposon mutants of *P. inhibens* DSM 17395 showed that residual wild type cells might still attach to the mutant strains. Therefore, mutant strains Tm#3163 and Tm#3902 were sequently streaked out three times on 0.5 marine broth (1/2 MB) medium supplemented with 120 µg/mL kanamycin. Afterwards the identity of the mutants and the absence of wild type cells was determined by isolation of genomic DNA (QIAGEN blood & tissue kit) and a sensitive PCR-assay with primers specific to the *luxR* gene (PGA1_c03880).

Arbitrary PCR for Transposon insertion sites verification

Insertion sites of transposon mutants were determined by Arbitrary PCR according to O'Toole and Kolter (Jogler *et al.* 2011, O'Toole and Kolter 1998) and subsequent sequencing of PCR products.

First arbitrary PCR primers:
P808 (GTTGATGCGAGTGATTTTGATGACGA)
P813 (GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT)
Second arbitrary PCR primers:
P810 (GCAAAGCAAAAGTTCAAAATCACCAA)
P815 (GGCCACGCGTCGACTAGTAC)
Sequencing primer:
P812 (ACCTACAACAAAGCTCTCATCAACC)
Insertion sites:
Tm#3163: 374972+
Tm#3902: 374869+

PCR for verification of correct insertion size of Transposon
Forward primer: P1903 (CAGTGGGTTGTGACGCTAGA)
Reverse primer: P1904 (AGGACCAGATTACGACCAGA)
The expected fragment size for the wild type fragment is 1021 bp, while the fragment size for the transposon mutants is expected to be
3022 bp long.

PCR program
The initial denaturation step was performed at 94°C for 30 sec. This was followed by 30 cycles of denaturation at 94°C, 30 sec, annealing at 56°C, 30 sec, elongation at 68°C, 1 min and one final elongation step at 68°C, 10 min.

SUPPLEMENTARY RESULTS

Arbitrary PCR identified the insertion sites of the EZ-Tn5 transposon within the genome of *Phaeobacter inhibens* DSM 17395. The PCR-assay independently demonstrated the presence of the EZ-Tn5 transposon in the *luxR* gene in both individual mutants by a size shift of 2,001 bp compared to the respective band of the wild type (Figure S1). The test also documented the absence of residual wild type cells in the mutant strains. The identity of the mutants was thus validated.

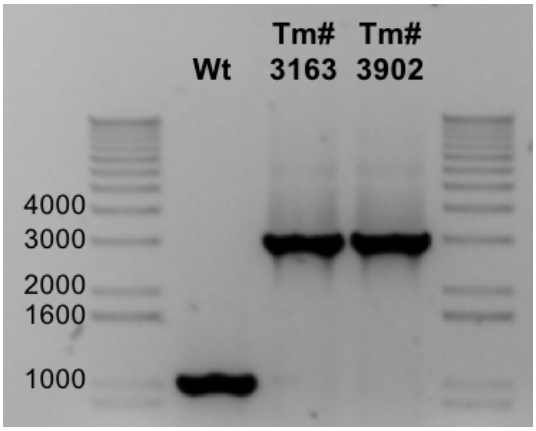

Figure S1: PCR results for verification of correct insertion of the EZ-Tn5 transposon in the *luxR* gene.
The fragments after PCR on wild type (Wt) DNA are 1000 bp long. The fragment size of the PCR products from the transposon mutants Tm#3163 and Tm#3902 are 3000 bp long.

Integrative Discussion and Outlook

The aim of this PhD study was to elucidate how Planctomycetes contrive to be successful in a variety of habitats. In this context, two hypotheses were considered and the results regarding future investigations and possible applications are discussed below.

Hypothesis I

According to the first hypothesis, the proposed lack of peptidoglycan (PG) was assumed to make Planctomycetes insusceptible against beta-lactam antibiotics, which might be produced by their potential competitors. This would represent a passive mechanism providing the ability to thrive in favorable habitats.

Commonly, prokaryotes belonging to the domain of bacteria can basically be divided into two types: Gram-positive and Gram-negative. These types were introduced based on their Gram stain retention in 1884 (Gram 1884). Both types differ in the divergent organization of their cell envelope. However, one of the main distinctions is the thickness and the localization of the PG-layer. Gram-positive bacteria possess a thick layer surrounding their cytoplasmic membrane, whereas the cytoplasmic membrane of Gram-negative bacteria is surrounded by a thin layer of PG and an additional outer membrane. Due to dissolved solutes within free-living microorganisms they are permanently exposed to significant osmotic pressure. Both Gram-negative and Gram-positive bacteria can counteract the osmotic pressure with their rigid PG cell wall aiding in maintenance of the cell shape and integrity. This makes PG one of the most conserved structural characteristics of bacteria. Only few bacteria were described not fitting the concept of Gram-positive or Gram-negative according to their PG nature. However, microorganism repeatedly make exceptions to this rule. For instance, bacteria of the genus Deinococcus stain Gram-positive due to the presence of a thick PG layer, but also possess an outer cell membrane. Thus, they are suggested as intermediates in the transition between Gram-positive and Gram-negative bacteria (Gupta 1998a).

However, since decades, bacteria belonging to the PVC-superphylum were described to represent a whole different exception. In particular, Planctomycetes (König *et al.* 1984, Liesack *et al.* 1986, Cayrou *et al.* 2010) Chlamydiae (McCoy and Maurelli 2006) and also Verrucomicrobia (Chin *et al.* 2001, Yoon *et al.* 2007a) were thought to lack PG completely. Members of the Chlamydiae are obligate intracellular bacteria, which are remarkably diverse, ranging from human and animal pathogens to symbionts of ubiquitous protozoa (Sixt *et al.* 2013). This intracellular lifestyle provides an osmotically stable environment which could make PG non-essential, as it is known for PG-less Mollicutes (Miles 1992). However, chlamydial species were known to be commonly sensitive to beta-lactam antibiotics which are targeting PG synthesis. Despite this hint, the presence of PG in chlamydial species was only verified a few years ago using state of the art technology (Pilhofer *et al.* 2013, Liechti *et al.* 2014).

Paradoxically, since Planctomycetes are known to be free-living bacteria and therefore are expected to need PG more than the osmotically protected Chlamydiae, many studies failed to detect PG (König *et al.* 1984, Liesack *et al.* 1986, Lindsay *et al.* 2001, Fuerst and

Sagulenko 2010). Initially, biochemical analyses did not detect the PG key components 2,6-diaminopimelic acid and *N*-acetylmuramic acid in isolated cell envelopes of eight planctomycetal strains (König *et al.* 1984). Instead they were proposed to possess a proteinaceous cell wall rich in proline and cysteine (Liesack *et al.* 1986). Further indicators, like the insensitivity to beta-lactam antibiotics (Cayrou *et al.* 2010) together with the biochemically determined negation of beta-lactamases (Claus *et al.* 2000) and the reported lack of several genes crucial for its PG biosynthesis (Jogler *et al.* 2012, Guo *et al.* 2014), reinforced the hypothesis of the absence of PG in Planctomycetes.

The assumed absence of PG facilitated a completely new interpretation of their cell organization. It is commonly acknowledged that they exhibit two membranes, as is the case for normal Gram-negative bacteria. Though historically, the outermost membrane was defined as a cytoplasmic membrane (Lindsay *et al.* 1997, Lindsay *et al.* 2001). This definition was mainly based on the absence of PG and the presence of RNA in the compartment between the two membranes. Since, RNA is not expected to be present in the periplasm (Lindsay *et al.* 1997, Lindsay *et al.* 2001). The cytoplasm was thus proposed to be separated into two compartments, the paryphoplasm and the pirellulosome, which are divided by the intracytoplasmic membrane (Lindsay *et al.* 1997, Lindsay *et al.* 2001). The compartmentalization into the two major regions by means of internal membranes was described manifold (Fuerst and Webb 1991, Lindsay *et al.* 1997, Lindsay *et al.* 2001, Niftrik *et al.* 2004, Fuerst 2005, Kulichevskaya *et al.* 2009, Lee *et al.* 2009, Santarella-Mellwig *et al.* 2010, Pinos *et al.* 2016). Further, anammox Planctomycetes were even found to possess an additional third membrane-enclosed compartment, the anammoxosome (Jetten *et al.* 2003, Lindsay *et al.* 2001, Niftrik *et al.* 2004).

Moreover, for the planctomycete *G. obscuriglobus* protein up-take from external medium associated with internal vesicle formation has been demonstrated (Devos *et al.* 2004, Lonhienne *et al.* 2010, Santarella-Mellwig *et al.* 2010). Hereby, proteins were incorporated only into the paryphoplasm compartment by a mechanism that was deemed to be analogous to clathrin-mediated endocytosis of eukaryotes. This finding was supported by the presence of clathrin-like membrane coat (MC) proteins in internal vesicles membranes (Santarella-Mellwig *et al.* 2010). Bioinformatic analysis revealed MC-proteins only in eukaryotes and members of the PVC-superphylum (Santarella-Mellwig *et al.* 2010). Since previously, endocytosis and compartmentalization was only observed for eukaryotes, Planctomycetes were suggested accordingly as 'missing link' between prokaryotes and eukaryotes, or as a species 'beyond the bacterium' (Forterre and Gribaldo 2010, Fuerst and Sagulenko 2011). However, the planctomycetal cell plan is under heavy debate. In the past years many lines of evidence pointed towards a more normal Gram-negative cell organization. In the meantime, the outermost membrane has been proposed to be a Gram-negative-like outer membrane based on the presence of marker genes for outer membrane proteins and lipopolysaccharide biosynthesis (Speth *et al.* 2012). As different independent studies came to congruent results the paryphoplasm should be interpreted as a

periplasm, the intracytoplasmic membrane as a cytoplasmic membrane and the pirellulosome as the cytoplasm (Santarella-Mellwig *et al.* 2013, Devos 2014a, Devos 2014b, van Teeseling *et al.* 2015, Boedeker *et al.* 2017).
Further evidence that Planctomycetes fit the concept of Gram-negative bacteria was given in chapter 2 of this thesis and in another independent study for anammox-Planctomycetes where the presence of PG, localized between outer membrane and cytoplasmic membrane, was shown (Jeske *et al.* 2015, van Teeseling *et al.* 2015).
The presence of the additional membrane-bound compartment, the anammoxosome, in anammox-Planctomycetes stays beyond controversy (Jetten *et al.* 2010, Lindsay *et al.* 2001, Niftrik *et al.* 2004).

One main argument in the reasoning that Planctomycetes lack PG was the above-stated insensitivity to beta-lactam antibiotics (Cayrou *et al.* 2010) combined with the determined negation of beta-lactamases (Claus *et al.* 2000). This argumentation could be revoked in chapter 2 as well. Observations made for *Staphylococci* revealed the used nitrocefin assay to be prone to false negative results (Pitkälä *et al.* 2007). Based on the identification of PG, this assay was evaluated to be possibly unreliable for Planctomycetes. Accordingly, a bioinformatic approach was successfully applied to demonstrate that various beta-lactamase genes were present in all investigated planctomycetal genomes.
Altogether, the finding of Planctomycetes possessing PG leads only to a partial refusal of the first hypothesis, since their Gram-negative cell organization might impart antibiotic resistance. Based upon a number of different observations including that the Gram-positive bacteria are the major reactors to antibiotics and that Gram-negative bacteria are, in general, resistant to them, it has been proposed that the outer cell membrane in Gram-negative bacteria evolved as a protective mechanism against antibiotic selection pressure (Gupta 1998a, Gupta 1998b, Gupta 2000, Gupta 2011). Thus, not the lack of PG would generate an advantage but rather the Gram-negative outer membrane in combination with the production of beta-lactamases would provide the selection advantage against antibiotics produced by potential competitors.

However, the finding of PG in planctomycetal cell walls poses new questions.
It is astonishing that established methods failed to identify PG in Planctomycetes. In chapter 2 complete cell sacculi resembling those of canonical Gram-negative bacteria were isolated employing a protocol modified from the original (König *et al.* 1984). Also the identified thickness of the PG layer fits the standards of other Gram-negative bacteria (Konorty *et al.* 2008). Moreover, using state of the art methods the key components could be identified in the same amount as in other Gram-negative bacteria (Jeske *et al.* 2015). From that perspective, it would be interesting to elucidate if yet unknown conditions make planctomycetal PG special compared to canonical PG. One possibility might be a different composition as it might be indicated by UPLC-analysis (van Teeseling *et al.* 2015).

Another uncertainty is the question of the completeness of the PG layer in Planctomycetes. Even though, complete intact planctomycetal sacculi were isolated, cryo-electron tomography of *P. limnophila* cells displayed local discontinuities of the PG layer (Jeske *et al.* 2015). However, it should be taken into account that a known artifact of electron tomography is the 'missing wedge' which appears due to the limitation of the tilting angle (Arslan *et al.* 2006). Thus this method is rather unsuitable for PG continuity studies. To completely exclude local or temporary discontinuities further investigations are needed. For example, sacculi from planktonic and sessile Planctomycetes could be compared to each other and also Planctomycetes in the budding process can be investigated for local discontinuities at the budding pole. For these projects it would be an advantage if the cultures would to be synchronized.

As a consequence of present PG, the planctomycetal endocytosis becomes questionable. Vesicles of 50 to 200 nm in size, which are thought to originate from the outer planctomycetal membrane by invagination (Lonhienne *et al.* 2010), would be blocked by a PG layer with a typical mesh size up to 2.0 nm (Yao *et al.* 1999). To confirm or revoke this objection, the above-mentioned questions have to be answered. Discontinuities or a yet unknown PG composition might someway facilitate vesicle based endocytosis in Planctomycetes. However, only recently evidence was given that Planctomycetes possess a periplasm space that can be greatly enlarged and convoluted (Boedeker *et al.* 2017). Presumably the resulting membrane invaginations of the adjustable cytoplasmic membrane were mistaken as vesicles. Nevertheless, extracellular macromolecules can be taken up and accumulate in the periplasmic space through yet unclear mechanisms (Boedeker *et al.* 2017). However, in this context the role and mode of action of the in Planctomycetes identified MC-like proteins needs further investigations. Also, the supposed presence of RNA in the formally entitled paryphoplasm, now as periplasm identified compartment becomes questionable and needs to be reanalyzed.

Hypothesis II

The second hypothesis assumes that Planctomycetes produce secondary metabolites (SM) with antibiotic effect which enables them to actively protect their habitats and resources against other faster growing bacteria.

First indications that Planctomycetes might have the potential to produce antibiotics are given by their lifestyle (Tekniepe *et al.* 1981) and their genomic organization (Jogler *et al.* 2012). Due to their phenotypic appearance they were, as *Actinobacteria*, initially thought to be fungi. Traits like their lifestyle switch after cell division, reminds of the cell cycle of myxobacteria. In regard of antibiotic production, both the Gram-positive Actinomycetes and the Gram-negative Myxobacteria belong to the yet best studied microorganism in the kingdom of bacteria (Berdy 2005, Blunt *et al.* 2013). Moreover, planctomycetal genomes were found to be particularly rich in extracytoplasmic function sigma factors (ECFs), with *G. obscuriglobus* being one of the most ECF-rich bacteria sequenced to date (Jogler *et al.* 2012). Therefore, their demonstrated complex transduction repertoire seems to resemble that known for pseudomonads, which also rank in the top ten of bacterial bioactive SM producers (Berdy 2005). Studies employing different bioinformatics tools found several microbial taxa with high biosynthetic potential amongst *Clostridium*, *Burkholderia*, *Pseudonocardia*, *Photorhabdus*, *Xenorhabdus*, *Chitinophaga* and *Herpetosiphon* also Planctomyces were identified as promising targets for novel bioactive molecules (Challinor and Bode 2015). Most of these promising genera are characterized by large genome sizes, which has been shown to correlate with an increased percentage of genomic capacity devoted to specialized metabolism (Cimermancic et al. 2014). Thus, the rather large planctomycetal genomes indicate the presence of SM genes as well. This together and the fact that their adaptation to a wide range of partly uncharted habitats, including extreme environments (Lage and Bondoso 2014), leads to the assumption that Planctomycetes developed diverse yet unexplored metabolisms with suspected novel bioactive molecules that await discovery (Jeske *et al.* 2013, Cimermancic *et al.* 2014, Graca *et al.* 2016,).

It is generally accepted knowledge that most SM related genes may be silent under axenic laboratory cultivation conditions (van Wezel and McDowall 2011, Gomez-Escribano and Bibb 2012, Yamada *et al.* 2012). Accordingly, the focus in chapter 3 is on the bioinformatic investigation if planctomycetal genomes harbor SM genes and thus have the potential to produce bioactive compounds of medical interest. In the second part of chapter 3, potential trigger substances are determined that could mimic certain environmental conditions that might facilitate the transcriptional activation of otherwise silent SM genes.

Bioinformatic analyses with antiSMASH (version 2 and 3.0.5) revealed many SMs in planctomycetal genomes. Except for small statistical outliers, the number of SM related genes correlated with previously observed correlation between genome size and number of such

genes (Donadio *et al.* 2007, Cimermancic *et al.* 2014, Müller and Wink 2014). Remarkably, all 15 analyzed planctomycetal genomes of this study and others (Aghnatios *et al.* 2015, Graca *et al.* 2016) harbored terpenes or terpene-like clusters which seem to be a common planctomycetal feature. As discussed in chapter 3, terpenes are a large and diverse class of organic compounds. A multitude of different terpenes and terpenoids, manly of plant or fungal origin, are known. As diverse as their appearance also their functions range from antibiotic to hormones over flavor-/ odor-constituents or pigments. Though, most important for commercial purpose are terpenes that act as vitamins or antitumor agents (Yamada *et al.* 2012). However, it is assumed that at least some terpene synthases in Planctomycetes are likely to be involved in carotenoid (tetraterpenoids) synthesis. First investigations employing UV-VIS and NMR spectroscopy of carotenoids, extracted from *P. limnophila*, revealed the presence of a 3,4-dihydrolycopene-like carotenoid with absorption maxima at 500 nm and 540 nm and an additional absorption maximum at 900 nm (unpublished). In nature carotenoids serve two key roles: they absorb light energy for utilization in photosynthesis complexes and they protect from photo damage (Armstrong and Hearst 1996). For *P. limnophila* it was anecdotally observed that liquid cultures exhibit a more intense pink shade if cultures were exposed to light or were cultured under increased oxygen conditions. Similar observations were made for strain Mal15^{T} (unpublished). Exposure to excessive light and oxygen increases the emergence of singlet oxygen and other reactive oxygen species (ROS) which can result in cell death. Thus, carotenoids in both species might function as antioxidants protecting against ROS. However, this observation needs further investigation to be verified. For example, in biochemical activity assays intact carotenoids from these Planctomycetes could be investigated for their ability to reduce ROS. Another possibility would be to knock-out the carotenoid coding genes in Mal15^{T} or *P. limnophila*. Subsequent the generated mutant cultures could be monitored for increased damage caused by ROS.

Also noticeable, eight of the 15 analyzed planctomycetal genomes harbored at least one bacteriocin-like gene. Most of the in Planctomycetes identified bacteriocins are probably homologues to cypemycin, microcin J25 and linocin M18 which are described in more detail in chapter 3. These ribosomally synthesized stable antimicrobial peptides were found to exhibit narrow to broad activity spectra (Cotter *et al.* 2013). They became of medical interest since they were frequently found to be produced by non-pathogenic bacteria that normally colonize the human body (*e.g. Lactobacilli*) (Nardis *et al.* 2013). This observation would apply very well to the finding of Planctomycetes being part of healthy human ecto-flora for example on facial skin (Hannigan and Grice 2013, Somboonna *et al.* 2017). However, the role of bacteriocins in microbial communities has not been well-established yet. Bacteriocins may serve as anti-competitor compounds enabling an invasion of a strain or species in an established microbial community (Lenski and Riley 2002, Riley and Gordon 1999) or act as communication molecules in bacterial consortia like biofilms (Gillor *et al.* 2008).

Another conspicuousness is that all analyzed genomes except from *P. staleyi* and *P. mikurensis* encode at least one thiotemplate modular systems (TMS) such as nonribosomal peptide synthetases (NRPS) and polyketide synthetases (PKS) which produce numerous small bioactive molecules of medical importance (Donadio *et al.* 2007). It was found that planctomycetal genomes encode different TMS clusters with similarities to other known TMS producers like *Bacillus* and *Microcystis* (Graca *et al.* 2016). However, several identified TMS clusters show indeed sequence similarity with TMS found in different Myxobacteria (Graca *et al.* 2016). For instance, a type I PKS cluster identified in the *R. baltica* SH1 genome was shown to presumably encode for an epothilone-like product which was previously isolated from *Sorangium cellulosum* (Graca *et al.* 2016). Suchlike observations were made for *R. brasiliensis* where a myxalamid pathway was predicted (Graca *et al.* 2016). Epothilones are a class of tubulin target agents effective against human ovarian and breast cancer (Cheng *et al.* 2008) whereas myxalamids are antibiotics known to be produced by *Myxococcus xanthus* (Gerth *et al.* 1983). Other Planctomycetes revealed pathways for the antibiotic stigmatellin produced by *Stigmatella aurantiaca* (Kunze *et al.* 1984) and also for myxothiazol which represents an antifungal agent from *Myxococcus fulvus* (Gerth *et al.* 1980, Graca *et al.* 2016). These findings reinforce the initial assumption that due to their Myxobacteria-resembling lifestyle Planctomycetes are likely to produce similar but yet distinct SMs. As described above for the terpenes, also the exemplarily TMS do not only vary in their structural appearance but also in their biological (or medical) function.

As mentioned in chapter 3, with the change of the antiSMASH version not only the number of identified clusters changed but also the cluster affiliation varied in several cases. This could be probably explained by improved algorithms. Moreover, discrimination especially in the case of TMS is still not trivial since even different types of hybrid clusters exist. Moreover, the planctomycetal genomic organization that seems to differ from the known bacterial genomic organization might be of topic. It is known for Planctomycetes that not all genes that are commonly involved in a process, are localized in an operon. For instance, in most bacteria and archaea the 16S- and 23S-rRNA along with the 5S-rRNA form an operon as they are found in clusters and are transcribed together. This is not the case for planctomycetal genomes where the 16S-rRNA seems to be commonly localized independently from 23S- and 5S-rRNA. Therefore, the assumption of 'guilt by association' cannot be necessarily applied for Planctomycetes. One possibility could be that two or more independent identified SM clusters might be involved in the production of one metabolite. Another assumption might be that one cluster could be involved in the modification of several products from different identified SM clusters. This would fit to the observation which was made regarding the above-mentioned tremendous amount of ECFs in Planctomycetes (Jogler *et al.* 2012). Leading to the conclusion that the planctomycetal regulation process has not yet been explored extensively enough to apply bioinformatic prediction tools like antiSMASH to get fully reliable results. However, this assumption might rule out the recent finding of a study employing a combination of different bioinformatic SM identification tools (antiSMASH

(Weber *et al.* 2015), BAGEL3 (van Heel *et al.* 2013) and PRISM (Skinnider *et al.* 2015)) which revealed Planctomycetes, based on their genome information, to be rather in the midfield of potential novel top SM producers (Tracanna *et al.* 2017). Nevertheless, it was also stated that the wide diversity of SM related genes is found in bacteria with small or middle number of SM genes and thus should not be discarded in the search for novel antimicrobials (Tracanna *et al.* 2017). In fact, to prioritize specifically for antimicrobials, it is necessary to go beyond the genome sequences and couple the genomic information to ecological and functional data (Tracanna *et al.* 2017).

Unfortunately, the major difficulty is that SMs are produced prevalently due to environmental cues, such as inter-organismic interactions (Seyedsayamdost *et al.* 2011, Straight and Kolter 2009). Thus to study SMs it is of importance to determine such stimuli that lead to the production activation under laboratory conditions. Another issue is that established methods for bioactive small molecule identification are based on knowledge of SMs secreted by high amount producers. These methods often relay either on the high amount or the high efficacy of the product or on spectroscopic properties of the yet known products. Thus the amount of a for instance slightly active antimicrobial compound produced by Planctomycetes might be too low for conventional detection methods or might be overlooked due to its missing fluorescent properties based on its structural features. However, with a slight modification this overlooked antimicrobial compound might be turned into highly active agent. To avoid this worst case scenario best possible for Planctomycetes, two basic approaches are implied in chapters 3 and 4. Based on the knowledge that Planctomycetes are frequently associated with eukaryotes, such as water plants or algae (Bengtsson and Øvreås 2010, Bondoso *et al.* 2011, Hempel *et al.* 2008, Matsuzawa *et al.* 2010, Pizzetti *et al.* 2011a, Pizzetti *et al.* 2011b) the second part of chapter 3 deals mainly with the identification of potential trigger compounds for Planctomycetes that might be secreted by such interaction partners. The Phenotype MicroArray technology is to a certain extent applicable for this purpose since the assay is based on quantifiable detection of utilization of defined compounds. The advantage is that not only substances used for the energy metabolism but also compounds that are utilized as trigger substances are detected by this assay. On the other side, further investigations are needed to deconvolute which compounds indeed serve as trigger substance and not only as carbon source for energy metabolism. On that account, the main focus in chapter 4 is the conception of consecutive tools to facilitate the discrimination between energy source and trigger substance. One main aspect is the introduction of the so-called Maintain Medium (MM) that provides all essentials to let Planctomycetes survive but lacks a carbon source. Employing the MM enables the investigation of single substances for stimuli properties. Moreover, techniques were established for the extraction of even low abundant SMs from planctomycetal liquid cultures, for the subsequent isolation of these compounds for further bioactivity assays and their structural elucidation (Jeske *et al.* 2016).

A further approach to enable the characterization of for instance naturally low abundant SM

from Planctomycetes might be based on genetic modification. However, ever since their discovery, Planctomycetes had been recalcitrant to genetic manipulation, thus precluding such investigations of their SM pathways. Therefore, analyses of SM profiles combined with gene inactivation studies would be very difficult to implement in every single species. Hence it would be easier to bypass the genetic manipulation of every single species and employ heterologous gene expression in a surrogate host to correlate the identified biosynthetic genes with metabolites. However, in case of Myxobacteria it turned out that the best product yields can be achieved in related host organisms (Fu *et al.* 2008, Julien and Shah 2002). In first transcriptome analyses it was shown that probably because of the high phylogenetic distance and eventually due to the lack of essential regulatory elements *Escherichia coli* is rather inappropriate as a surrogate host for planctomycetal gene expression studies (Olga Jeske 2013, Master thesis). Problems could multiply in further metabolome analysis for example due to missing precursors and different codon usage. However, genetic tools to manipulate *P. limnophila* and most recently three other Planctomycetes (*Gemmata obscuriglobus*, *Gimesia maris*, and *Blastopirellula marina*) were developed (Jogler *et al.* 2011, Erbilgin *et al.* 2014, Rivas-Marin *et al.* 2016). In case of *P. limnophila*, the proof-of-principle was given by constructing a mini Tn5 transposon containing a kanamycin resistance cassette together with *gfpmut2* under the control of the endogenous and constitutive glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter from the *P. limnophila* genome. The construct was transferred by electroporation to *P. limnophila* and integration into its chromosome was verified. Wide-field epifluorescence microscopy revealed GFP expression in the recipient *P. limnophila* cells (Jogler *et al.* 2011, Boedeker *et al.* 2017). In addition, a protocol was developed for site-directed mutagenesis by homologous recombination in *P. limnophila* (Rivas-Marin *et al.* 2016, Boedeker *et al.* 2017). Accordingly, *P. limnophila* could be a more suitable host for heterologous gene expression of planctomycetal SM genes than *E. coli* is. In some cases, it might prove useful to replace the constitutive promoter against an inducible promoter to allow time-regulated high level production of even toxic compounds. As Planctomycetes show high resistance to most antibiotics an antibiotic induced promoter system like the tetracycline-controlled transcriptional activation (Gossen *et al.* 1995) would be applicable for Planctomycetes. However, one should keep in mind that high-titer production of SMs can only be achieved if there is no bottleneck in trigger signal delivery and precursor supply, like CoA-activated short chain carboxylic acids in terms of polyketide biosynthesis (Donadio *et al.* 2007). Hence, also studies on the function and regulation of primary metabolic routes should be done.

Worth mentioning is that not only planctomycetal SM with antibacterial function could be studied in this way. Examples mentioned above indicate that for instance also novel antitumor (epotholine-like) or antifungal (myxothiazol) agents might be discovered from Planctomycetes. However, it should be taken into account that also novel SMs that are maybe originally identified to be 'inactive' might have some kinds of inherent activities which have not yet been discovered. Only the methods to detect their possible, perhaps until now

unknown type of activity, have to be developed. There is no reason to suppose that natural products should not exhibit some kind of biological function (Berdy 2005). This also includes SM that provide the ability of bacterial interaction with other organisms.

Taken together, first results from this thesis and from independent studies reinforce the second hypothesis that Planctomycetes produce SM with antimicrobial activity to compete with other bacteria for resources. However, the assumption of high potential is to date mainly based on bioinformatic analyses. Thus far, compounds which displayed antibiotic activity at comparable high concentration against used sentinel bacteria have been isolated from planctomycetal cultures (Graca *et al.* 2016, Jeske *et al.* 2016) but their structures have not been elucidated. However, it has to be considered that the tested sentinel bacteria are less likely to interact with Planctomycetes in nature and thus do not represent the main targets for the by Planctomycetes produced compounds. Moreover, the role of antibiotics produced by bacteria is not utterly resolved. In the past, great effort has been put into identification and development of new compounds that effectively target bacterial viability. The concentrations of these compounds required to achieve an antimicrobial effect are likely high compared to the concentrations in which these compounds can be found in natural settings (Yim *et al.* 2007). According to this argumentation line bacteria from aquatic environments were excluded in advance from being promising antibiotic producers. Because, due to dilution effects in aqueous environments it seems to be unlikely that they produce antimicrobial compounds that probably never reach the required concentration. However, how low concentrations of antibacterial compounds affect bacterial survival remained unresolved in this context. Pioneering studies on the global transcriptome response of dedicated pathogens to sub-inhibitory concentrations of antibiotics have demonstrated that these molecules can indeed affect the expression of genes related to stress response, colonization, virulence, motility or biofilm formation (Yim *et al.* 2007, Skindersoe *et al.* 2008, Richards and Melander 2009, Romero *et al.* 2011). This observation has prompted the idea that antibiotics might actually act as signal molecules in natural environments, facilitating intra- or inter-species interactions within microbial communities (Davies 2006). In fact, chapter 5 deals with the proposed conjecture of antibiotics in low concentrations acting as quorum-sensing (QS) mediators.

Before bacterial communication systems have been investigated in detail it has been observed that certain bacterial communities exhibit cooperative behavioral patterns. The swarming motility and biofilm formation of microorganisms like *Pseudomonas* and *Vibrio* provide excellent examples of multicellular behavior among bacteria (Fuqua *et al.* 1994). To date the best known bacterial communication pattern is QS of Gram-negative bacteria. The process relies on the production, release, and group-wide detection of signal molecules called autoinducer, which in Gram-negative bacteria are typically acyl–homoserine lactones (AHL) (Fuqua *et al.* 1994, Nealson 1977). The detection and gene activation is regulated by the generally called LuxI/LuxR system or lux box (Fuqua *et al.* 1994). The lux box represents a

conserved regulatory sequence in Gram-negative bacteria (Callahan and Dunlap 2000, Hao *et al.* 2010) but trough structural differences in the aliphatic tails of the AHLs they are able to discriminate between species specific autoinducer signals (Fuqua *et al.* 1994, Fuqua and Greenberg 2002, Ng and Bassler 2009, Romero *et al.* 2011).

In chapter 5 it is shown that the addition of stieleriacine A_1 in different concentrations affects the *Stieleria maiorica* culture in diverging ways. Low concentrations of stieleriacine A_1, which were ascertained to be the physiological concentration in the original cultures, reduced significantly the lag phase duration and thus might accelerate biofilm formation. Whereas, a hundred-fold higher concentration lead to decreased division activity and to increased cell death. Aside from that, stieleriacine A_1 also affects significantly biofilm formation of two *Roseobacter* species. *Sulfitobacter dubius* and *Phaeobacter inhibens* are both of marine origin and are known to be associated with algae (Buchan *et al.* 2005, Frank *et al.* 2014) and thus are very likely to interact with marine algal associated Planctomycetes in natural settings. Interestingly, *P. inhibens* is known to produce the antibiotic tropodithietic acid (TDA) and a growth hormone presumed to promote algal growth (Wang *et al.* 2016). Nothing comparable is known for *S. dubius*. Planctomycetes however are insensitive to TDA. Strikingly, the presence of stieleriacine A_1 reduces the biofilm formation ability of *S. dubius* but enhances biofilm formation of *P. inhibens*. In Δ*luxR* mutants of *P. inhibens* the effect of stieleriacine A_1 seems to be even enhanced compared to the wild type. Since *P. inhibens* possess only one *luxR* homologue (Berger *et al.* 2011) this result point towards a mechanism that is not relaying on the lux box known from other Gram-negative bacteria. This assumption is supported by the fact that the *S. maiorica* genome possesses different *luxR* encoding domains but *luxI* genes do not appear to be present. As utilization of stieleriacine A_1 as carbon source was excluded in both experimental settings, it seems to have another target at least in *S. maiorica* and *P. inhibens*. However, this assumption and the actual mode of action of stieleriacine A_1 have to be verified in further studies. For example, in transcriptome studies of these bacteria treated with stieleriacine A_1. Nevertheless, these first findings may allow the theory that the secreted stieleriacine A_1 might act as inter- and intra-species lure. On the one hand, it might be that motile Planctomycetes sense stieleriacine A_1 that was secreted by other sessile Planctomycetes which inhabit a favorable habitat. As a consequence, the motile Planctomycetes might switch from motile cells into sessile cells, start reproduction and thus magnify the planctomycetal biofilm. On the other hand, simultaneously, also other bacteria that may improve the planctomycetal biofilm community are attracted whereas bacteria that would not provide advantage to the planctomycetal community are repelled by the same molecule. According to the results in chapter 5, it might be that these other species are not affected until a critical number of Planctomycetes and thus a high stieleriacine A_1 concentration has been reached. Accordingly, *P. inhibens* would not only provide antibiotic protection against planctomycetal competitors but also foster their food source. If this theory could be confirmed, it would be a strong evidence for the original hypothesis that secondary metabolites produced by Planctomycetes help them to actively

protect their habitats, despite stieleriacine A_1 itself exhibits only slight antibiotic activity.
Even though the LuxI/LuxR system along with AHLs as autoinducer in Gram-negative bacteria seems to be conserved (Callahan and Dunlap 2000) it is conceivable that also QS systems mediated through yet unknown autoinducer like stieleriacine A_1 might exist. In Gram-positive bacteria for example, QS molecules are usually modified small peptides or amino acids, which are sensed by membrane-associated kinases (Miller and Bassler 2001). On top of this, recently, a communication system based on electrochemical signals, comparable to that of animal nerve cells, has been discovered for bacteria (Prindle *et al.* 2015). Remarkably, not only neighboring cells but entirely separate colonies can coordinate their actions in this way (Prindle *et al.* 2015). From that point, results of chapter 5 might even allow the assumption of a yet unknown QS system discovered from Planctomycetes.

A recent report by the World Health Organization revealed that antibiotic resistance is a major threat to public health (WHO 2014). Treating bacterial infections with antibiotics has two major drawbacks. First, since antibiotic therapies rely on resulting death of invading bacteria and their clearance from the body, they place a strong selective pressure on bacteria to develop resistance mechanisms (Delago *et al.* 2016). The second major drawback is that many antibiotics are not selective for one particular microorganism and will also kill or harm commensal bacteria, with potentially devastating effects on the delicate balance of the microbiome (Delago *et al.* 2016).
QS-controlled genes and functions are diverse and affect mostly species-specific bacterial behaviors like proliferation and cell maintenance, including sporulation and bacterial competence but also cell motility or adhesion and antibiotic production. Moreover, cell community behaviors like biofilm formation and dispersal, bioluminescence as well as host colonization are initiated by QS molecules (Fuqua *et al.* 1994, Whitehead *et al.* 2001, Jimenez *et al.* 2012, Monnet *et al.* 2016).
In the search for novel treatment approaches the concept of antivirulence was introduced. Antivirulence aims to prevent virulence functions and behaviors including biofilm formation and does not directly target the viability of the pathogens (Cegelski *et al.* 2008). Thus, interference of bacterial QS is a main point of antivirulence approaches (Shapiro and Wencewicz 2016). In particular, the understanding of the mechanism of biofilm formation and dispersal seems to be an important and promising leverage point.
Biofilms are ubiquitously present, usually found on solid substrates submerged in or exposed to an aqueous solution or humid environment, although they can also form floating mats on liquid surfaces (Hall-Stoodley *et al.* 2004). Some biofilms are formed by single species but they may contain as well different types of pro- and eukaryotic microorganisms (Hall-Stoodley *et al.* 2004, Nadell *et al.* 2009). Biofilms are remarkably difficult to treat with chemicals and antibiotics as the agents may be inactivated by or fail to penetrate into the biofilm (Lewis 2001). Bacteria within biofilms exhibit an up to thousand-fold increased

resistance to antimicrobial compounds, compared to same bacteria under non-biofilm conditions (Obst *et al.* 2006, Stewart and Costerton 2001). According to a record of the National Institutes of Health (NIH) in 2002 it is estimated that biofilm forming bacteria are responsible for over 80% percent of all microbial infections in the human body. Thus, knowledge about the mechanisms of biofilm formation and dispersal could lead to tremendous improvement of medical treatments of biofilm associated diseases. However, the area of possible application for QS interference extends not only to medicine but also to applied domains like agronomy and water engineering (for review see (Fitridge *et al.* 2012)). For example, applications against biofilm-associated biofouling which poses serious technological and economical problems as in naval transportation, aquaculture, the petroleum industries, bioreactors or water distribution networks and wastewater plants are conceivable (Fitridge *et al.* 2012, Grandclement *et al.* 2016).

QS interference has two major advantages. While antibiotic therapies place a strong selective pressure one key advantage proposed in targeting QS is based on the premise that a treatment that does not suppress growth of a cell will not exert a selective pressure to develop resistance to that treatment (LaSarre and Federle 2013). QS is not an essential process, and QS mutants in general have not displayed growth defects. Nevertheless, interfering with the regulation of virulence factor production will likely reduce fitness for survival in certain situations. However, the selective pressure would be dramatically reduced, which could help promote long-term efficacy of anti-QS therapies (LaSarre and Federle 2013).

The second advantage is that unlike most antibiotics, QS signals are mostly species specific (Fuqua *et al.* 1994, Jimenez *et al.* 2012, Delago *et al.* 2016) and thus probably would not harm other essential bacteria in the environment of application.

Granted, QS interference would presumably not achieve the same game-changing breakthrough in microbial treatment as antibiotics had when they were launched, as the ultimate clearance of bacteria from the body still depends on a healthy immune-system. Nevertheless, identification of potent new QS inhibitors, with maximal specificity and potency, could provide a whole generation of new therapeutics with a broad range of applications, while at the same time providing good tolerability for the treated patients or the environment.

Closing words

Results presented in this thesis together with results for anammox-Planctomycetes

(van Teeseling *et al.* 2015) have unveiled Planctomycetes as Gram-negative bacteria. However, knowledge obtained in this project does not only provide a better understanding of planctomycetal cell biology but does also form the basis for developing promising bio-controlled strategies for directed screening on Planctomycetes for bioactive secondary metabolites. Moreover, the first novel secondary metabolite derived from Planctomycetes has been investigated. Stieleriacine A_1 is suggested to be a yet unknown quorum-sensing molecule that might be promising in future terms to treat or prevent bacterial infestation associated with biofilm formatio

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- Albert Einstein

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- J. W. von Goethe

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Curriculum Vitae

Olga Jeske was born on 21th July 1987 in Bolek, Kazakhstan. She graduated from the Jacobson-Gymnasium, Seesen, Germany in 2007. Following this, she studied Biology at the Technische Universität Carola-Wilhelmina zu Braunschweig from 2008-2013, where she obtained her Bachelor of Science as well as her Master of Science degree *summa cum laude*. During her studies she participated in the selection committee for new students at the TU Braunschweig and supervised various internships for Bachelor students in plant cell biology.

In the course of her biology education, she performed research projects at the institute for Plant Biology (TU Braunschweig, Germany), the department of Forest Genetics and Plant Physiology (UPSC, Sweden), the department of Microbial Agents (HZI, Germany) and the junior research group of Microbial Cell Biology and Genetics (DSMZ, Germany). In 2013 she started her PhD project, of which the results are described in this thesis, at the DSMZ under the supervision of Dr. Christian Jogler, with Prof. Dr. Mike Jetten as her promotor. During her PhD, Olga was PhD spokesman from 2014-2015 and also completed her apprenticeship as CMAS Scientific Diver in 2015.

Starting June 2017, Olga started working as a post-doc at the department of Microbial Ecology and Diversity under supervision of Prof. Dr. Jörg Overmann to study the functionality of mangrove ecosystems.

Publications

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